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The Neuroimmune Axis in Hip Osteoarthritis and Periprosthetic Inflammation

Manuel Ribeiro da Silva

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The Neuroimmune Axis in Hip Osteoarthritis and Periprosthetic Inflammation

Tese de Candidatura ao grau de Doutor em Medicina, submetida à Faculdade de Medicina da Universidade do Porto.

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LIST OF ABBREVIATIONS

ADRs	adrenergic receptors
AL	aseptic loosening
BDNF	brain-derived neurotrophic factor
β2-ARs	β2-adrenergic receptors
BI	whole blood
BMP	bone morphogenetic protein
C	controls
CGRP	calcitonin gene related peptide
CoCr	cobalt chromium
DALYs	disability-adjusted life years
DNA	deoxyribonucleic acid
EDS	energy dispersive spectroscopy
ELISA	enzyme-linked immunosorbent assay
FGFs	fibroblast growth factors
G-CSF	granulocyte-colony stimulating factor
GC	multinucleated giant cells
GM-CSF	granulocyte macrophage colony stimulating factor
H&E	hematoxylin & eosin

HOA	hip osteoarthritis
HPA	hypothalamus-pituitary-adrenal
IC	immune cells
IFN	interferon
IL	interleukin
IL1-ra	IL-1 receptor antagonist
iNOS	inducible nitric oxide synthase
LIF	leukemia inhibitory factor
LL	lining layer
M-CSF	macrophage-colony stimulating factor
MMPs	matrix metalloproteinases
MoM	metal on metal
MoP	metal-on-polyethylene
mRNA	messenger ribonucleic acid
MT	Masson’s trichrome
NE	norepinephrine
NGF	nerve growth factor
NI	no information
NPY	neuropeptide Y

OA	osteoarthritis
OPG	osteoprotegerin
OSM	oncostatin-M
PDGF	platelet-derived growth factor
PE	polyethylene (PE)
PGE2	prostaglandin E2
PMMA	polymethyl methacrylate
PMN	polymorphonuclear leukocytes
PP	polymeric particles
qRT-PCR	quantitative real-time polymerase chain reaction
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor kappa-B ligand
RNA	ribonucleic acid
RT	room temperature
S	serum
SEM	scanning electron microscopy
SEMA	semaphorin
SF	synovial fluid
SLL	sublining layer

SP	substance P
TGF	transforming growth factor
TH	tyrosine-hydroxylase
THA	total hip arthroplasty
TIMPs	inhibitors of metalloproteinases
TNF	tumor necrosis factor
Tuj-1	neuron-specific class III ß-tubulin
UHMWPE	ultra-high-molecular-weight polyethylene
VEGF	vascular endothelial growth factor
Y1R	neuropeptide Y Y1 receptor
YLDs	years of life lived with disability
ZrO2	Zirconium dioxide

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LIST OF PUBLICATIONS

The work performed in the frame of this thesis resulted in the following publications:

1. Neuroimmune expression in hip osteoarthritis: a systematic review. Manuel Ribeiro da Silva, Daniela Linhares, Daniel Vasconcelos, Cecília Juliana Alves, Nuno Neves, Gilberto Costa, Meriem Lamghari. BMC Musculoskelet Disord. 2017 Sep 11;18(1):394. doi: 10.1186/s12891-017-1755-2.

2. Immune response and innervation signatures in aseptic hip implant loosening. Daniel Vasconcelos*, Manuel Ribeiro da Silva*, António Mateus, Cecília Juliana Alves, Gil Costa Machado, Joana Machado Santos, Diogo Paramos de Carvalho, Inês S. Alencastre, Rui Henrique, Gilberto Costa, Mário A. Barbosa, Meriem Lamghari. J Transl Med. 2016 Jul 7;14(1):205. doi: 10.1186/s12967-016-0950-5.
** equal contribution*

3. Interplay between sympathetic signaling and inflammation in aseptic loosening of hip joint replacement. Manuel Ribeiro da Silva, Daniel Vasconcelos, Inês S. Alencastre, Maria José Oliveira, Daniela Linhares, Nuno Neves, Gilberto Costa, Rui Henrique, Meriem Lamghari*, Cecília Juliana Alves*. Sci Rep. 2018 Oct 8:16044. doi: 10.1038/s41598-018-33360-8.
** equal contribution*

ABSTRACT

Hip osteoarthritis is a leading cause of disability in the population, and its burden is expected to increase as a result of its raising incidence. Hip osteoarthritis is associated with progressive destruction of the cartilage, and although a definitive characterization of the pathways implied in the process of cartilage injury is still not clear, the role of inflammation in hip osteoarthritis pathogenesis, progression and severity is becoming evident, due to the actions of pro inflammatory cytokines and proteinases in the hip, responsible for cartilage degradation, without rescue responses that can compensate this process.

Total hip arthroplasty is a highly cost effective surgery in patients with hip osteoarthritis refractory to non-operative treatment. Developments in implants materials and production are responsible for increased survival of total hip arthroplasty in the long term. Nevertheless the rate of revision arthroplasty is expected to increase significantly in the upcoming years. Aseptic loosening is the main cause for failed arthroplasty and revision surgery. This process is caused by numerous and very small particles released from implants stimulating the release of pro-inflammatory and pro-osteoclastic cytokines that simultaneously increase the number, survival and activity of osteoclasts and inhibit osteoblasts.

Neurotransmitters and peripheral nervous system have been described to be involved in the pathophysiology of osteoarticular diseases, namely inflammatory diseases. Sensory and sympathetic nerve fibers have an important role in chronic inflammation regulation and bone resorption, by influencing the pro-inflammatory and anti-inflammatory cytokines

concentrations and actions. However we know little regarding their actions in the development of hip osteoarthristis and even less in the failure of total hip arthroplasty.

The main objective of this PhD Thesis was to investigate the involvement of the neuroimmune axis in the biological events leading to hip osteoarthritis and aseptic loosening.

First, we assessed the nervous and immune system profile of patients with hip osteoarthritis when compared to healthy controls. We performed the first systematic review on neuroimmune expression in hip osteoarthritis patients using data from studies in humans, allowing an integrated approach of the changes caused by hip osteoarthritis, both at a local and systemic level. Our review highlights the lack of a clear analytical profile of hip osteoarthritis, both systemically and locally. We found deregulation in the balance of pro-inflammatory versus anti-inflammatory cytokines. Regarding the influence of the neuroimmune axis in the biological events leading to hip osteoarthritis, only two articles were found relating neuropeptides expression to the pathogenesis of hip osteoarthritis, which is representative of the lack of available information in this area.

Second, we characterized the immune and innervation profiles of hip aseptic loosening patients when compared with patients with hip osteoarthritis. This was the first study performing this analysis in the hip of human patients, and brought a new insight into the role of the neuroimmune axis in hip osteoarthritis and aseptic loosening patients. Our results demonstrate differences both in the immune response and local innervation of hip osteoarthritis when compared to aseptic loosening patients. Both present

sensory innervation, with different distribution between both groups, but there is an absence of sympathetic nerve fibers in aseptic loosening tissues when compared to osteoarthritis. This is a distinct feature in the innervation profile in these patients and the first time it was described.

Finally, we characterized the impact of aseptic loosening in the activity of the sympathetic nervous system. Following the results described in our previous work, we evaluated the systemic and local profile of neuroimmune molecules involved in the interplay between the sympathetic nervous system and the periprosthetic inflammation in hip aseptic loosening. We confirmed that the usual effectors for the sympathetic anti-inflammatory action were absent in macrophages in aseptic loosening patients, but present in the osteoarthritis group. These findings are in favor of a local reduction of the anti-inflammatory effects performed by the sympathetic system, and the perpetuation of a pro-inflammatory environment in aseptic loosening patients. Systemically we found no impact of these changes in the Hypothalamus-Pituitary-Adrenal axis activity, which leads us to consider that in humans these pathological responses occurring at the hip are mostly confined to the joint.

Overall, under the scope of this thesis, we described a new pattern of innervation in aseptic loosening, characterized by the absence of sympathetic activity. This impairment occurs locally at the hip, affecting both adrenergic and Neuropeptide Y-ergic signaling, without systemic translation. This finding highlights the local sympathetic signaling as a putative target to mitigate the inflammatory response to debris release and consequently extending the lifespan of orthopedic implants.

RESUMO

A osteoartrite da anca é uma das causas mais importantes de incapacidade na população, sendo expectável um aumento do seu impacto socioeconómico, resultado da sua crescente incidência. A osteoartrite da anca está associada a uma destruição progressiva da cartilagem, e embora os mecanismos implicados neste processo não estejam totalmente esclarecidos, o papel da inflamação na sua patogénese, progressão e gravidade tem-se tornado evidente, decorrente da acção de citocinas pró-inflamatórias e proteinases na anca, responsáveis pela destruição da cartilagem, perante a ausência de mecanismos de compensação eficientes.

A artroplastia total da anca é um procedimento altamente custo efectivo em doentes com osteoartrite da anca refractários ao tratamento conservador. Desenvolvimentos nos materiais e na produção de implantes têm sido responsáveis por um aumento na sobrevida das artroplastias totais da anca a longo prazo. No entanto, é expectável que as taxas de artroplastia de revisão da anca aumentem de forma significativa nos próximos anos. O descolamento asséptico é a principal causa para falência desta artroplastia e cirurgia de revisão. É um processo causado pelas numerosas partículas de pequenas dimensões, libertadas pelos implantes, que estimulam a libertação de citocinas pró-inflamatórias e pró-osteoclásticas, que simultaneamente aumentam o número, sobrevida e actividade dos osteoclastos e inibem os osteoblastos.

Os neurotransmissores e o sistema nervoso periférico têm sido descritos como estando envolvidos na patogénese de doenças osteoarticulares, particularmente nas doenças inflamatórias. As fibras sensitivas e simpáticas

têm um papel importante na regulação da inflamação crónica e da reabsorção óssea, ao influenciarem a concentração e ação das citocinas pró e anti-inflamatórias. Contudo, o conhecimento acerca do seu papel no desenvolvimento da osteoartrite da anca e na falência das artroplastias totais da anca é ainda limitado.

O principal objectivo desta Tese de Doutoramento foi investigar o papel do eixo neuroimune no desenvolvimento da osteoartrite da anca e no descolamento asséptico.

Inicialmente avaliamos o perfil nervoso e imune de doentes com osteoartrite da anca quando comparados com controlos saudáveis. Realizámos a primeira revisão sistemática sobre expressão neuroimune em doentes com osteoartrite da anca, utilizando dados sobre estudos em humanos, permitindo uma abordagem integrada das alterações causadas pela osteoartrite da anca, tanto a nível local como sistémico. A nossa revisão enfatiza a falta de um perfil analítico claro da osteoartrite da anca tanto a nível sistémico como local. Relatamos a existência de uma desregulação no balanço de citocinas pró *versus* anti-inflamatórias. Relativamente ao papel do eixo neuroimune no desenvolvimento de osteoartrite da anca, apenas dois artigos foram encontrados, evidenciando a escassez de informação disponível nesta área.

De seguida caracterizámos o perfil imune e de inervação em doentes com descolamento asséptico da anca quando comparados com doentes com osteoartrite da anca. Este é o primeiro estudo disponível que realiza esta análise em doentes humanos, revelando uma nova informação sobre o papel do sistema neuroimune na osteoartrite da anca e no descolamento asséptico. Os nossos resultados demonstram diferenças na resposta imune e inervação

local de doentes com osteoartrite da anca quando comparados com descolamento asséptico. Ambos apresentam evidência de inervação sensitiva, mas com distribuição diferente entre os grupos, verificando-se uma ausência de fibras simpáticas no grupo com descolamento asséptico quando comparado com osteoartrite da anca. Esta é uma característica distinta da inervação entre estes grupos, tendo sido a primeira vez que foi descrita.

Por último, caracterizámos o impacto do descolamento asséptico na actividade do sistema nervoso simpático. Seguindo os resultados descritos no nosso trabalho anterior, avaliámos o perfil sistémico e local das moléculas neuroimunes envolvidas na relação entre sistema nervoso simpático e inflamação periprotésica no descolamento aséptico da anca.

Confirmámos que os efetores habituais da ação anti-inflamatória do sistema simpático estavam ausentes nos macrófagos de doentes com descolamento asséptico, mas presentes nos do grupo de osteoartrite da anca. Estes achados são a favor de uma provável redução dos efeitos anti-inflamatórios locais por parte do sistema nervoso simpático, e de uma perpetuação do ambiente pró-inflamatório em doentes com descolamento asséptico. Sistemicamente não encontrámos qualquer impacto destas alterações ao nível da actividade do eixo hipotálamo-hipófise-suprarrenal, o que nos leva a considerar que no humano estas alterações patológicas na anca estão essencialmente confinadas à articulação *per se*.

Em resumo, nesta tese, descrevemos um novo padrão de inervação no descolamento asséptico, que se caracteriza pela ausência de actividade simpática. Esta alteração verifica-se ao nível da anca, afectando o sistema adrenérgico e neuropéptido Y-érgico, sem tradução sistémica. Estes

resultados realçam a possibilidade do sistema nervoso simpático local ser usado como um possível alvo terapêutico para minimizar a resposta inflamatória aos resíduos libertados, e consequentemente, aumentar a sobrevida dos implantes ortopédicos.

CHAPTER I

GENERAL INTRODUCTION
AIMS OF THE THESIS

Hip osteoarthritis (HOA) is a leading cause of disability in the elderly, with radiographic signs present in more than 5% of people over 65 years old (1). The risk of developing symptomatic HOA is more than 25% in population that lives up to 85 years and the risk of undergoing total hip arthroplasty (THA) is estimated at 10% for general population (2). This makes HOA one of the most common and important joint disorders.

Impact of Hip Osteoarthritis

Society burden associated with HOA is expected to increase significantly as a result of its raising incidence. This is related with the increase in life expectancy and the subsequent aging of population, together with the increase of diseases with impact in joint degeneration, such as obesity, as well as the lack of effective disease-modifying drugs active in HOA (3, 4). HOA is known to have a significant impact on quality of life, causing severe pain and disability. This is reflected in multiple reports on the increase of disability-adjusted life years (DALYs) and on years of life lived with disability (YLDs) observed from 1990 to 2010 (3). Actually, HOA is currently one of most worrisome diseases reported in the Global Burden Disease 2010 (3).

HOA burden is reflected not only on the individual but also on a society level. It is responsible for decreased productivity, premature leave from the workplace, loss of autonomy, increased needs for healthcare services, personal assistance and institutionalization. Salmon et al estimated annual direct costs from 0.5 to 10.9 thousand-euros/year per patient and indirect costs from 0.2 to 12.3 thousand-euros/year per patient (4). The weighted

average annual costs were 11.1, 9.5 and 4.4 thousand-euros/year per patient for total, direct and indirect costs, respectively.

Biological Background of Hip Osteoarthritis

Osteoarthritis encompasses a mixed group of joint disorders that have a common presentation with decreased range of motion and pain. In particular, HOA is associated with synovial inflammation, followed by progressive destruction of the cartilage in a nonuniform fashion, subchondral bone thickening and finally formation of osteophytes (1, 2).

For a long period HOA was considered a “wear and tear” disease. However the increasing evidence of synovitis in the early phases of this disease raised the possibility that both inflammation and the immune system could be involved in the development and progression of HOA (5).

The pathogenesis of HOA is far from being clearly established (1, 6). Biomechanical factors have been implicated in this process. Repetitive stresses at the cartilage surface can cause molecular and cellular changes related to HOA development. Inappropriate mechanical loading to the hip joint leads to cartilage injury by degradation of the matrix macromolecules and decreased expression of proteins necessary for normal joint function, such as aggrecan proteoglycan and type II collagen. In response there is an increase in the synthesis of other proteoglycans and type I collagen, characteristic of immature cartilage. This shift in cartilage phenotype results in a matrix that is not able to preserve normal hip function (7, 8). Together these can lead to an increase in apoptotic cellular response and in the release of pro-inflammatory cytokines, as nitric oxide (2, 7, 8). However, a definitive characterization of

biochemical pathways and signalling molecules implied in the process that drive cartilage injury is still not clear (6).

In the beginning of osteoarthritis (OA) development, cartilage reacts in an attempt of an early repair, with chondrocyte proliferative, increased production of cartilage matrix, and a simultaneous increase in catabolic cytokines and matrix-degrading enzymes. The lesion progresses with a reduction of the tensile forces in the cartilage matrix, caused by cleavage of type II collagen and loss of proteoglycans (9). Pro inflammatory cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)-α have a central role in this process, sustaining the inflammatory process and targeting chondrocytes, stimulating them to secret catabolic enzymes, what enhances cartilage destruction (9, 10). Although the mechanism is yet to be found, increased levels of these cytokines have been found in OA synovial fluids, even in the absence of infiltration of macrophages and neutrophils into joint tissues (11).

Synovitis and lymphocytic infiltration have also been reported in early-stage OA (2). The fibroblast- and macrophage-like synovial cells, may produce catabolic cytokines in response to breakdown products from the damaged cartilage, and are potential sources of cytokines that could induce chondrocytes to synthesize and secrete cartilage-degrading proteases and other cytokines and proinflammatory mediators (11).

Role of Inflammation in Hip Osteoarthritis

Several authors reported the presence of inflammation in synovial tissue of patients with HOA. This is characterized by inflammatory and immune cell

infiltration, cytokine secretion, hyperplasia of synovial lining cells and activation of resident cells (5, 12).

Macrophages in the lining layer (LL) and T cells in the sublining layer (SLL) are the most abundant cells. Mast cells (in the SLL and around blood vessels), B cells and plasma cells were also found, although in smaller amounts (13). Also, different cytokines associated with immune cells have been detected in OA synovial tissue (5, 14). These are indicative of a role played by inflammation in HOA pathogenesis, progression and severity.

Cytokines have a proven physiological function in normal tissues, being essential at low concentrations for normal homeostasis (11). In HOA this balance is compromised, and cytokines are present in increased levels, with distorted ratios, acting on resident cells in a paracrine-autocrine fashion (11).

As previously referred IL-1 and TNF-α play a central role in HOA pathophysiology. In vitro and animal model studies demonstrated that IL-1-β stimulates the expression of other pro-inflammatory cytokines as IL-6 and TNF-α (15). It also inhibits the function of extracellular matrix proteins growth factors. TNF-α presents similar effects to, and is synergistic with IL-1 in the process of cartilage destruction (16, 17). Other identified catabolic and pro-inflammatory cytokines include IL-8, IL-17, IL-18, oncostatin-M (OSM) and leukemia inhibitory factor (LIF) (18).

Inhibitory cytokines that block the action of catabolic cytokines were found in synovial fluid, which was interpreted as an attempt to control the catabolic process (11).

These anti-inflammatory factors include IL-1 receptor antagonist (IL-1ra), interferon (IFN)-α, IL-4, IL-10, IL-11 and IL-13 (19). Other factors that oppose

this destructive cartilage process were also found in vitro, as insulin-like growth factor-I, transforming growth factor (TGF)-β /bone morphogenetic protein (BMP) family, and fibroblast growth factors (FGFs) (20). These are cartilage anabolic factors. Other factors as IL-6 and TGF-β may have dual roles (11).

An imbalance in both proteinases and inhibitors produced by cartilage cells, which influence cartilage destruction was also reported. Matrix metalloproteinases (MMPs) are the best-known factors implied in degradation of proteoglycans and collagens and were found in cartilage and synovial samples from OA patients (21). In reaction to local increased levels of these active MMPs, inhibitors of metalloproteinases (TIMPs) are also increased (22). However, this raise is neither sufficient, nor effective, to counteract the high levels and actions of MMPs.

This process creates a vicious circle, where degradation products and fragments of cartilage released in the joint cavity trigger local inflammatory response by the synthesis of catabolic cytokines, which perpetuate and amplify cartilage destruction (11).

Moreover, the adaptive response, with increased expression of type II collagen and repair of matrix components, is limited and adult mature chondrocytes cannot regenerate the normal cartilage matrix architecture (11, 16).

Total Hip Arthroplasty Evolution and Survival Rates

THA is a very effective treatment for patients with refractory pain and significant disability. It is currently indicated in patients with HOA presenting with substantial discomfort and functional impairment, upon failure of conservative treatment. Nevertheless, the best timing for joint-replacement surgery is not yet established (1).

Although the first studies showed a significant failure rate soon after the surgery (1% a year over a period of 10 years), posterior development in implants' design, materials and fixation techniques overcame this problem, with modern implants presenting a long survival rate after implantation (1, 23). Nowadays, more than 1 million THA are performed annually. THA has been demonstrated to be a highly cost effective surgery in patients refractory to non operative treatment (2).

THA implants consist of femoral and acetabular components that can be fixed in different ways. The available systems are usually modular and consist of independent femoral head, femoral stem, acetabular shelf and acetabular liner. The fixation can be achieved by the use of cement or by bony growth onto or into the porous implant surface (24).

THA survival in the long term is related to the amount of debris released by the bearing surfaces and the longevity of component fixation (25, 26). This is one of the main spotlights in the designing and development of arthroplastic implants and their fixation, with efforts being made to improve their survivorship and function. These advances are responsible for the current 98% survivorship at 10 years, and 93% survivorship at 25 years of modern cemented stems (25). Also for cementless femoral components similar

survival rates have been reported. They present comparable survival at 10 years (95–100%), but a slightly better survival at 15 years (85–94% versus 70–95%) (26). Although both cementless and cemented femoral implants present excellent long-term survival rates, the cementless implants are currently the first choice for the majority of surgeons (23, 24).

The technological development of modern non-cemented stems has included the use of metal alloys that are more biologically inert and less stiff, and the use of porous coatings. A trend towards an increased modularity has also been seen, allowing the possibility of different head sizes and offset options for the same stem, as well as different bearing surfaces for the femoral head (24). These developments displayed a huge array of intra-operatory solutions with a unique stem that is both more similar in elasticity to the bone and has optimal bone ingrowth properties (23, 24).

New metals have also been developed, with the emergence of highly porous metal alloys (trabecular metal and tantalum) that are not only more porous and similar to bone, but can also be manufactured in different sizes and shapes, increasing the available solutions for both primary and revision surgeries (26). In fact, their demonstrated soft tissue and bone ingrowth proprieties led to an increase in their use (23, 24, 27).

Osteolysis, associated with wear debris from polyethylene, motivated the development of new bearing surfaces including cross-linked ultra-high-molecular-weight polyethylene (UHMWPE) (28). This proved to be more resistant then the standard ones, and became the most frequently used bearing surface worldwide (28).

Other new combinations besides the metal on polyethylene have also been developed.

Ceramic on ceramic or ceramic on highly cross-linked polyethylene articulations, became popular, as they present less friction and produce a small number of wear particles. Despite the absence of longer follow-ups, 10-year studies on ceramic on ceramic and ceramic on polyethylene demonstrated a significant success of these pairs (29). With the development of new ceramics, with diminished risk of fracture, the utilization of ceramic on ceramic or ceramic on highly cross-linked polyethylene is expected to be popularized in the upcoming years (24, 29).

Metal-on-metal bearing surfaces also present diminished friction, with low wear rates and particle production. Clinical studies with intermediate follow up (5-10 years) in young patients have demonstrated good results, but concerns regarding the systemic effects of released and absorbed ions were reported, as their role in organ failure or carcinogenic pathways are not yet known, and have halted the early popularity of this option (24, 30).

Revision Total Hip Arthroplasty: burden and complications

Even with these improvements in implants survival, the rate of arthroplasty revision is expected to grow over the next 20 years by 137% (31, 32).

Revision THA represents a challenge, and is associated with re-revision rates of 15.8% at 1 year, as described by Badarudeen et al (31). The risk of complication in the second procedure is significantly higher than in the first revision (31, 33, 34). Moreover patients that undergo revision surgery are progressively more complex, with increased comorbidities, increasing even

more the rate of adverse events (33). The mortality risk in these cases is rising, opposing the tendency in primary THA (33). Previous works reported mortality rates of 0.87%-2.6% in the first 6 weeks after the revision THA surgery (31, 33, 34). Other serious complications in these patients include infection (up to 17,3%), re-revision (15.8%), venous thromboembolic disease (11.1%), dislocation (5.43-14,4%), pulmonary embolism (3.24%), and death (2.11%) (31, 33, 34).

The main factor for failed primary THA in both mid and long term is aseptic loosening, responsible for about 70% of the revisions (23, 25). Aseptic loosening is a low-grade inflammatory process, where a macrophage driven process leads to bone resorption, implant loosening and arthroplasty failure (35). It is usually asymptomatic for years and is preceded by periprosthetic osteolysis, due to progressive destruction and disappearance of the bone that contacts the implant (35). Rates for osteolysis were reported in 5-20%, but these numbers are expected to increase significantly, because nowadays we are doing more THA procedures overall, especially in younger patients (40% of patients submitted to THA are below 65 years). (32). Younger patients, with increased life expectancy, are more probable to experience osteolysis and aseptic loosening throughout their life (36).

THA longevity is affected by several factors including gender, age and surgeon's skills (36). The conditions pre dating the primary arthroplasty can also have an effect in the occurrence of aseptic loosening. Accordingly, male patients below 40 years old, and those with sequelae of congenital diseases are more prone to this type of failure. Also, uncemented implants might be less affected than cemented implants (36).

The ability to reduce the number of revision procedures would have a significant impact both in patient outcomes, and on the healthcare system.

Periprosthetic Inflammation and Aseptic Loosening

The recent improvements in THA function and durability are related to the utilization of cross linked UHMWPE, cobalt chromium (CoCr), and alumina ceramics that contribute to a positive balance between friction, lubrication and wear (32).

In normal circumstances, the host reacts to all types of implanted biomaterials with a foreign-body response. When it comes to definitive implants this response is aberrant and continued, aiming to isolate the foreign material from the surrounding tissue, achieved by coating the tissue-material interface with a collagenous and avascular membrane. The initial step in this process is a complex inflammatory process interaction at the tissue-blood-material interface (37).

The inflammation is the body's normal protective response to any injury (including surgery) or foreign bodies. In this process, vascular permeability is increased by neuropeptides, as calcitonin gene related peptide (CGRP) and Substance P (SP), followed by the attraction of neutrophils and other polymorphonuclear leukocytes (PMN) to the injury site, and subsequent recruitment and activation of macrophages (38, 39). Macrophages assemble at the implant site and lead to further production of chemo-attractive signalling molecules such as platelet-derived growth factor (PDGF), TNF-α, IL-6, granulocyte-colony stimulating factor (G-CSF), and granulocyte macrophage colony stimulating factor (GM-CSF), that perpetuate this process (40). In the

final stage of this response, a chronic inflammation status ensues, characterized by infiltration of macrophages and fibroblasts, as well as neovascularization within the new-formed tissue (granulation tissue), that is a precursor for the future fibrous capsule (32, 41). This is a protective and physiological response from the host to a foreign body, as is the case with an arthroplasty. It is completely different from the response to wear particles released by different biomaterials.

Wear debris and particles from THA implants released due to fatigue, adhesion and excessive abrasion in the articulating interfaces of the implants components, can initiate important immunological reactions, that eventually cause implant failure (32).

William Harris was one of the first to describe this phenomena and called it “particle disease”, highlighting the importance of the particles generated by the implants in the development of a deleterious host response (42). The amount of bone destruction and resorption is, in part, dependent on the origin, size and amount of the prosthetic particles that reciprocally condition the severity and depth of the deregulated osteolytic response (41, 42). In particle disease, numerous and very small particles (less than micrometers in size) released from the implants cause periprosthetic cells to produce pro-inflammatory/pro-osteoclastic cytokines that simultaneously increase the number, survival and activity of osteoclasts and inhibit osteoblasts (41).

The joint fluid produced by the synovial-like membrane composed of macrophages and fibroblasts, facilitates the spreading of particles across the joint. The fluid not only washes the particles from the articulating area, but also transports the signalling and inflammatory molecules to the adjacent

bone areas (41). This explains why particle disease can expand and be found in different sites, leading to a spread and expansion of osteolysis, with consequent progressive deterioration of the bone-prosthetic interface (41).

Wear particles from UHMWPE are responsible for the recruitment and activation of macrophages within the tissue, producing inflammatory factors as GM-CSF, macrophage-colony stimulating factor (M-CSF), PDGF, MMPs, prostaglandin E2 (PGE2), IL-6, IL-3, IL-1 and TNF-α (40, 43). This contributes to a local inflammatory and osteolytic environment surrounding the implant (43). Both IL-1 and TNF-α contribute to the activation, survival and differentiation of osteoclasts (44). Local fibroblasts also contribute to the bone resorption and destruction by promoting the formation of osteoclasts, through the expression of the receptor activator of nuclear factor kappa-B ligand (RANKL) (45). The macrophages attempted response at degrading these particles by phagocytising them, is ineffective because these are both biologically inert and non-degradable (46). Thus the macrophages keep releasing the aforementioned cytokines and mediators, exacerbating and perpetuating the osteoclastic process (32, 41).

UHMWPE wear particles osteolytic potential is dependent on both particle size and volume. Particles in the size range of 0.1–1.0 μm are more biologically active than larger particles in terms of osteolytic cytokine release (46). The currently used crosslinked UHMWPEs produce smaller particles (<0.1 μm) in large numbers (47). They have a smaller wear volume than the one from the conventional THA, but can be systemically disseminated, with UHMWPE wear particles being found in the lymph nodes, spleen and liver (48). These particles trigger a different biological response than the original

ones, their effect in the local osteolysis being unclear, as well as if they are associated with any kind of systemic effects like the ones that happen with metallic particles/ions (49). Although they appear to be inactive in stimulating proinflammatory cytokine release both locally and systemically (49), few reports are yet available.

Besides crosslinked UHMWPEs, other materials, such as metals and ceramics have been developed as low wear alternative bearing surfaces to avoid particle-induced osteolysis (49).

Metals like CoCr produce large amounts of nanometer-sized particles through wear and corrosion (50). Their large number and small size have increased risk of cytotoxic, genotoxic and immunological impact in both local and distant organs, as they can be absorbed and were already found systemically in bone marrow, lymph nodes, liver and spleen (48). They cause increased concentration of free radicals and Chromium concentrations, with effects in both cell death and mutagenesis properties, through damage of intracellular organelles and deoxyribonucleic acid (DNA). Clinical studies of surrounding tissues on metal on metal (MoM) implants have demonstrated increased levels of translocations, aneuploidy and DNA damage (51). However no evidence between the use of these prosthetic materials and cancer occurrence was found in previous epidemiological studies (52). MoM implants have been demonstrated to cause less osteolysis than implants using UHMWPE, because most of the wear particles identified in these implants are less than 100 nm in size, and thus much smaller than the UHMWPE particles (50). In these cases ostelysis is usually related to patient's metal hypersensitivity (32, 41).

Ceramics are the hardest biomaterial implant developed for THA, with a wear rate 1000-fold lower than UHMWPE-Metal implants (53). It is also highly durable and has little or none known toxic effects (54). In normal conditions their debris range from 5–25 nm. A greater number of particles and debris, with sizes of 14–70 μm particles, can occur in cases of damage to components due to localized overloading or scuffing in cases of mal alignment or trauma (54).

The research comparing the biological response of ceramic wear particles with UHMWPE and metal particles is limited. Nevertheless it is unlikely that the necessary amount of particles needed to create the same responses can be achieved in vivo, considering the wear rates reported for ceramics (32).

Taking in consideration that patients having the same kind of surgery and implants, with comparable radiographic alignment and physical activity, do not present the same responses as the ones described above, reinforces the possibility than some kind of individual susceptibility to periprosthetic osteolysis can exist, despite the fact that its risk factors and pathways are still unclear (41).

Influence of the Neuroimmune Axis in Joint Diseases

The processes previously described have been explained by detailing the individual events that are involved in both HOA and THA failure, specifically at a local level in the hip joint. Despite the great progresses made in the characterization of these events, the systemic impact of local events, as well as their regulation, remains unclear.

In previous years, attention has been given to the role of the neuroimmune axis and neurogenic stimuli in the interaction and interrelation between immune cells and cytokines with different systems and environments (55). Current literature demonstrates the influence of neuroimmune axis and neurogenic stimuli in inflammation and cytokines expression, immune cells proliferation and migration, matrix synthesis and degradation, angiogenesis and connective tissues synthesis (56).

These advances in research raised questions regarding the influence of the neuroimmune axis in the pathogenesis of both hip osteoarthritis and aseptic loosening.

Over the past decades, accumulated evidence has pointed to the role of peripheral nervous system and its neurotransmitters as critical players in the pathophysiology of several joint articular diseases (26, 27). In healthy joints, under physiological conditions, sensory and sympathetic nerve fibers have been found in bone, periosteum and synovial tissue, but there are no reports of their presence in healthy cartilage (57). However, despite the lack of innervation, cartilage and joint development are modulated and influenced by neurotransmitters released either from nerve fibers located in neighbouring tissue, or directly from chondrocytes (57). SP, CGRP and sympathetic neurotransmitters affect chondrogenic differentiation and metabolism of chondroprogenitor through specific receptors for sensory neuropeptides, neurokinin 1 receptor, CGRP receptor and sympathetic β2-adrenoceptor (57).

Under pathological conditions, the joint displays different patterns of innervation. Rheumatoid arthritis (RA) is characterized by changes in the density of sensory and sympathetic nerve fibers in the synovial tissue (58),

with an increase in SP nerve neurotransmitters together with a decrease in CGRP, adenosine and norepinephrine concentrations (58).

In the early stages of OA both sensory and sympathetic nerve fibers migrate into the cartilage. With OA development, an increase of sensory fibers when compared to sympathetic fibers is found, with both SP and CGRP nerve fibers present in a balanced way (59). This differs from RA, with a preponderance of SP fibers and a marked reduction of CGRP and sympathetic fibers (58, 59).

Sensory and sympathetic nerve fibers have an important role in chronic inflammation regulation in RA, as SP is known to have a pro-inflammatory role by increasing the concentrations of IL1, IL6 and TNF (60), while CGRP and sympathetic neurotransmitters present a anti inflammatory role by inhibiting these cytokines actions (61, 62).

Saxler et al first reported the increased presence of CGRP and SP nerve fibers in the articular cartilage of symptomatic patients with HOA, when compared to controls (63). Suri et al reported the same in the knee, and described that the nerves are in perivascular localization (64). Salo et al indicated that SP and CGRP presence precedes cartilage degeneration (65).

SP is up-regulated in response to mechanical stimulus in OA patients, what might indicate a role of this peptide in the maintenance of cartilage function and integrity (66).

In another hand, SP has been reported to play a major role as strong effector in the inflammatory response under the development of OA (57, 67). Increased SP in adult synovial fluid in OA patients was associated with catabolic effects on articular cartilage, and this increase could be promoted by TGF-β and FGF (67). These catabolic effects are related to an increase in IL-

1βn, TNF-α and MMPs concentrations and a simultaneous reduction in proteoglycans deposition (68).

CGRP actions are currently less well known, with contradictory information in the literature regarding its actions in chronic inflammation. Fox et al describe an anti-inflammatory by inhibiting macrophage activation and action (69). Dirmier et al presented a diminished presence of CGRP in synovial tissues of RA patients as a factor for a pro inflammatory status, describing the actions of CGRP as anti-inflammatory (58), while Walsh et al suggest CGRP as a factor contributing for pain and inflammation in chronic arthritis, by augmenting the release of SP and pro-inflammatory cytokines from macrophages (70).

Takeshita et al found a significant increase in sensory innervation and pro-inflammatory cytokines, as TNF-α, in HOA patients, when compared with non-OA patients (71). According to this study, TNF-α is released from nerve fibers besides macrophages and T cells, with a role in inducing neural ingrowth in addition to its known inflammatory proprieties (71).

Sympathetic nerve fibers have also been associated with physiological processes as bone remodelling, metabolism, mineralization, osteogenesis, and matrix differentiation (67). Moreover, they have been implied in the development of OA, by influencing bone and cartilage differentiation, remodelling and regeneration capacity, as well as eliciting significant effects in both osteoclasts and osteoblasts activity (57, 67).

The activation of the sympathetic nervous system in the context of inflammation results in the release of high amounts of sympathetic neurotransmitters, such as neuropeptide Y (NPY) and norepinephrine (NE),

known to induce an anti-inflammatory effect in a context-dependent manner (72).

The mechanism of anti-inflammatory action of sympathetic nervous system in arthritis is not completely understood, but possible mechanisms have been described in animal models. Stimulation of β2-adrenergic receptors (β2-ARs) on B cells by catecholamines, results in increased IL-10 production from these cells, and diminished proinflammatory activity by inhibiting IL-7 receptor (73)

In THA aseptic loosening, previous reports have mentioned the involvement of sensory nerve fibers in the regulation of the inflammatory processes and bone resorption, through local release of neuropeptides (74-76). In these cases, SP and CGRP nerve fibers were identified in the interface membrane of AL patients (75). SP was shown to raise local levels of IL-1 and TNF-a (76), with subsequent increase in osteoclastic response. However, we know little regarding the actions of the sensory nervous system and even less regarding the sympathetic nervous system.

Aims of the Thesis

The main objective of this PhD Thesis was to investigate the involvement of the neuroimmune axis in the biological events leading to hip osteoarthritis and aseptic loosening.

Thereby, the following specific objectives were defined:

1) *Assess the nervous and immune system profile of patients with hip osteoarthritis when compared to healthy controls*

We performed the first systematic review assessing the nervous and immune system profile, both at a local and systemic level, of patients with hip osteoarthritis when compared to healthy controls.

The results are presented in Chapter II:

"Neuroimmune expression in hip osteoarthritis: a systematic review."

da Silva MR, Linhares D, Vasconcelos D, Alves CJ, Neves N, Costa G, Lamghari M.

BMC Musculoskelet Disord. 2017 Sep 11;18(1):394. doi: 10.1186/s12891-017-1755-2.

2) *Characterize the immune and innervation profiles of hip aseptic loosening patients when compared with patients with hip osteoarthritis*

We investigated the local tissue inflammatory response, sensory and sympathetic innervation as well as associated local mediators in hip joint microenvironment underlying hip aseptic loosening when compared to osteoarthritis.

The results are presented in Chapter III:

"Immune response and innervation signatures in aseptic hip implant loosening."

Vasconcelos DM, Ribeiro-da-Silva M*, Mateus A, Alves CJ, Machado GC, Machado-Santos J, Paramos-de-Carvalho D, Alencastre IS, Henrique R, Costa G, Barbosa MA, Lamghari M.*

J Transl Med. 2016 Jul 7;14(1):205. doi: 10.1186/s12967-016-0950-5.

** equal contribution*

3) *Characterize the impact of aseptic loosening in the activity of the sympathetic nervous system*

We evaluated the systemic and local profile of neuroimmune molecules involved in the interplay between the sympathetic nervous system and the periprosthetic inflammation in hip aseptic loosening.

The results are presented in Chapter IV:

"Interplay between sympathetic signaling and inflammation in aseptic loosening of hip joint replacement" M. Ribeiro-da-Silva, D.M. Vasconcelos, I.S. Alencastre, M.J. Oliveira, D. Linhares, N. Neves, G. Costa, R. Henrique, M. Lamghari, C.J. Alves.

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CHAPTER II

NEUROIMMUNE EXPRESSION IN HIP OSTEOARTHRITIS: A SYSTEMATIC REVIEW

Article 1

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Neuroimmune expression in hip osteoarthritis: a systematic review

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Abstract

Background

Neuroimmune axis is central in the physiopathology of hip osteoarthritis (OA), but its specific pathways are still unclear. This systematic review aims to assess the nervous and immune system profile of patients with hip osteoarthritis (OA) when compared to healthy controls.

Methods

A systematic review followed PRISMA guidelines was conducted. A two-step selection process was completed, and from 609 references 17 were included. The inclusion criteria were: original articles on adult patients with hip OA, with assessment of neuroimmune expression. Articles with other interventions prior to analysis and those without a control group were excluded.

Results

Thirty-nine relevant neuroimmune markers were identified, with assessments in bone, cartilage, synovial membrane, synovial fluid, whole blood, serum and/or immune cells. GM-CSF, IFN-γ, IL-1α, IL-6, IL-8, IL-1 and TNF-α presented variable expression among tissues studied when compared between hip OA and controls. VEGFs and TGF-ß isoforms showed similar tendencies among tissues and studies. On nervous expression, CGRP, Tuj-1 and SP were increased in synovial membrane. Overall, patients with hip OA presented a higher number of overexpressed markers.

Conclusions

For the first time a systematic review on neuroimmune expression in patients with hip OA found an upregulation of neuroimmune markers, with deregulated balance between pro and anti-inflammatory cytokines. However, no clear

systematic pattern was found, and few information is available on nervous expression. This highlights the importance of future research with clear methodologies to guide the management of these patients.

Keywords: Hip osteoarthritis; Neuroimmunomodulation; Inflammation; Cytokines

Background

Hip osteoarthritis (OA) is a common chronic health condition and a leading cause of pain and disability among adults, impacting many health outcomes (1). The complex and multifactorial nature of hip OA is nowadays under the spotlight, and recent studies proposed a switch of the paradigm from a simple "wear and tear" to a much more complex mechanism, in which inflammatory mediators play a pivot role in initiation and progression of the pathologic process (1, 2).

Neuroimmune axis is known to control the development and perpetuation of multiple inflammatory diseases (1, 3). Immune cells and secreted cytokines have been established as important players in OA (4). Also, neuropeptides were recently proposed as critical molecules in the modulation of the inflammation and pain associated with OA (5). Recent works showed that each joint should be seen as an individual organ, with OA being not exclusively a disorder of articular cartilage, but also an organ failure, involving the whole joint with additional abnormalities especially in bone, ligaments, synovium and joint capsule (6-8). In particular, the understanding of the role of the nervous system, immune cells and cytokines in the pathophysiology of OA of the hip joint, and their association with the different clinical features of the disease is still limited (4, 9).

Although many studies are available on particular aspects of the role of immune system in pathologic mechanisms in hip OA (10), there are still no consistent reports, and no data is available on the general profile of neuroimmune expression in these patients. Few studies have addressed the cytokine profile in hip OA, and even those, focus only on a small set of

cytokines and in a limited range of samples (blood, bone, cartilage or synovial tissue). Moreover, the global picture of hip OA neuroimmune expression is yet to be defined. Therefore, there is a critical need for enlightening on the role of neuroimmune mediators produced at the hip joint in OA patients. This knowledge would be of utmost importance in the ongoing study of pathologic pathways underlying hip OA and an important step in the development of disease-specific modifying therapies.

This systematic review aims to characterize the local and systemic expression of neurochemical and immune biomarkers in patients with hip OA when compared to healthy controls.

Methods

Literature Search

A systematic search was performed in Pubmed using as main search terms: “neuroimmunity”, “osteoarthritis” and “hip”, and other equivalent terms. The limits used were a) English, French or Portuguese language, b) publication date from 2000 to March 2015, c) studies performed in humans, d) exclusion of reviews, editorials and comments.

Article Selection

Study selection was conducted in two phases (Figure 1). In Phase 1, two investigators screened the titles and abstracts independently. If one of them included the abstract, it was allowed into the Phase 2. In Phase 2, full-text articles were analyzed independently, and disagreements were discussed between reviewers. Inclusion criteria were: 1) original data; 2) data on neuroimmune expression; 3) patients with hip OA; 4) adults (>18 years old). Exclusion criteria were: 1) studies performed in tissues other than the hip; 2) participants with known main diseases other than hip OA, e.g. rheumatoid arthritis; 3) patients or samples submitted to intervention prior to the analysis that may influence the results; or 4) absence of a control group. When manuscripts or data were not available, the authors were contacted. One study was excluded because results on cytokine expression were outside the range described by the manufacturer of the technique use and no answer on clarification from the author was received until the end of data analysis.

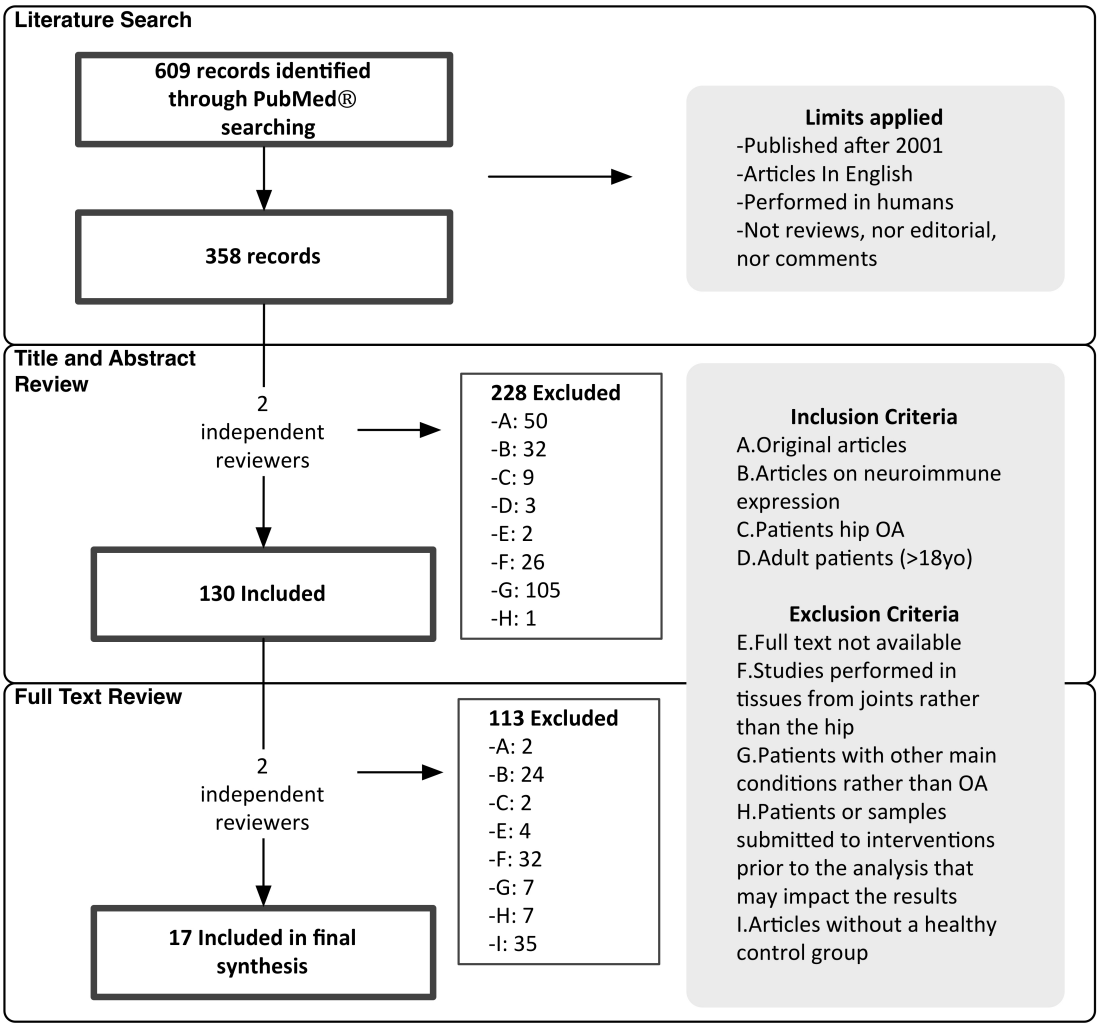

Figure 1. Selection Process

Data Extraction

Data was extracted using an electronic form developed by the authors and general article information is on Table 1.

The study group was defined as patients with hip OA, upon a diagnosis based on clinical, radiological and/or histological analysis. Controls were defined as healthy patients without OA diagnosis (hip or another).

Studies were grouped based on the technique used for neuroimmune expression measurements, namely: Bead-based multiplex immunoassay, Enzyme-Linked Immunosorbent Assay (ELISA), quantitative real-time

polymerase chain reaction (qRT-PCR) or immunostaining; and based on the sample used: synovial fluid, synovial membrane, cartilage, whole blood (blood), serum, immune cells, or bone. In each individual subgroup values were compared between patients with OA and controls (Supplementary Table 1).

Author, year	Sample Size	No		Sample collection	Age		Sex (No of men)		Definition of:	
		OA	*C*		*OA*	*C*	*OA*	*C*	*OA*	*C*
Pombo-Suarez, 2009 [20]	22	11	11	Surgery	NI	NI	NI	NI	Clinical, imagiological and histological	Non-OA hip fracture
Koorts, 2012 [16]	26	7	19	Puncture	NI	NI	NI	NI	Clinical and imagiological	Normal X-ray; No inflammation
Granchi, 2006 [12]	128	39	20	Puncture	56 (13)	60 (12)	13	14	Clinical and imagiological	Clinically healthy donnors
Granchi, 2003 [13]	112	22	39	Puncture	60 (3)	52 (3)	8	15	Clinical and imagiological	Clinically healthy donnors
Hashimoto, 2013 [14]	35	7	3	Surgery	52.7 (37–73)[a]	28 (15–44)[a]	5	4	Clinical, imagiological and histological	No tissue degeneration
Abrams, 2014 [10]	34	17	17	Puncture	59.2 (11.9)	38.3 (11.1)	9	5	Clinical and imagiological	Normal X-ray
Pape, 2000 [19]	105	22	20	Puncture	47 (19)	22-26[b]	12	20	Clinical	NI
Dallos, 2009 [11]	NI	NI	NI	NI	NI	NI	NI	NI	Clinical and imagiological	NI
Hulejova, 2007 [7]	85	55	30	Surgery/Puncture	56.4 (10.6)	67.5 (9.5)	9	8	Clinical and imagiological	Healthy; No OA on X-ray; No inflammation
Shi, 2002 [22]	NI	12	10	Surgery	71 (8)	NI	2	4	Clinical and imagiological	No metabolic disease; No OA diagnosis; No OA on surgery
Kumarasinghe, 2012 [17]	NI	5	5	Surgery	67 (12)	81 (6)	0	0	Clinical and imagiological	Non-OA femoral neck fracture?
Lavigne, 2004 [18]	NI	19	11	Surgery	NI	NI	NI	NI	Clinical and imagiological	NI
Saxler, 2007 [5]	6	3	3	Surgery	74	65	0	0	Clinical and imagiological	No pain or degeneration
Sanchez-Sabaté, 2009 [21]	35	16	11	Surgery	66.9 (7)	42.3 (11.2)	7	7	Clinical and imagiological	No metabolic disease
Takeshita, 2012 [23]	62	50	12	Surgery	61.3 (48–80)[a]	79 (63–90)[a]	4	3	Clinical and imagiological	No clinical or imagiological OA
Verdier, 2005 [24]	9	6	3	Surgery	69-85[b]	76-86[b]	9	9	Clinical and imagiological	Healthy patients with hip fracture
Hopwood, 2007 [15]	35	24	21	Surgery	49-85[b]	43-85[b]	10	10	Clinical and imagiological	No bone disease

Table 1. Description of the sample and methods of the studies included and hip OA assessment
All as Mean (Standard Deviation), unless otherwise indicated. OA Osteoarthritis, C Controls, NI No Information. [a]Mean (range). [b]Min-Max

Data analysis

Data was gathered on the significance of the comparisons, with a significant statistical value being defined as $p<0.05$.

Ratios on neuroimmune expression between hip OA and control patients were computed, and a visual illustration with arrows was assembled, grouped by tissue sample (Table 2). If different measurement techniques resulted in different ratios, these were all displayed. A general immune expression pattern was also displayed for each tissue (Figure 2).

Only 2 studies were available for the same immune marker when grouped by tissue and technique, and no meta-analysis was performed, since high heterogeneity was predictable.

This systematic review follows the PRISMA recommendations and PRISMA checklist was completed and is available on Supplementary Table 2 (11).

Results

Articles' search retrieved a total of 609 references. After limits applied 358 were included in the final review. In the first selection phase, 228 articles were excluded, mainly studies with patients with other known conditions than hip OA (Figure 1). In the second selection phase, all but 4 full-text articles were retrieved and analyzed. Seventeen studies were included in the systematic review (5, 9, 12-26). Twelve were cross-sectional studies, three cohorts, and one a case-control. Apart from one, all studies primary goal was neuronal and/or immune expression analysis. All were hospital-based studies, with outpatient clinic recruitment. Sample sizes ranged from 6 to 128 participants (Table 1).

Most studies (n=14) based OA diagnosis on clinical and radiological evaluation; one only had a clinical diagnosis, and two also had a histological analysis. Controls definition was highly variable and mostly based on clinical examination and X-ray. Individuals with non-OA hip fracture were used as healthy controls in three studies (Table 1).

Thirty-nine relevant neuroimmune markers were identified from the studies retrieved, and data on the comparison between patients with hip OA and controls was gathered (Supplementary Table 1). Their expression was evaluated by five different laboratorial techniques. All but three articles reported in vivo results. The tissue samples studied in the included reports were bone, cartilage, synovial membrane, synovial fluid, whole blood (blood), serum and immune cells (Supplementary Table 1).

Six articles did not present numerical values on the analysis performed. Data on general results and significance of the comparisons was gathered when possible and presented (Supplementary Table 1).

Fifteen articles reported on immune markers expression and two articles presented results on neurochemical expression. Only one study was available on neuroimmune expression in synovial fluid and in serum. Two were available on immune cells production and blood expression, 3 in synovial membrane, 4 in cartilage and 7 in bone. One article studied more than one tissue, with different techniques (Supplementary Table 1 and Table 2).

The following markers showed a different variation on neuroimmune expression in different tissues (Table 2):

- IFN-γ increased in synovial fluid, increased production in immune cells, decreased in bone;
- IL-6, increased in synovial fluid, blood and bone, decreased production in immune cells;
- TNF-α, increased in synovial membrane, cartilage, serum and blood, decreased in synovial fluid and decreased production in immune cells;
- IL-10, increased in synovial membrane and cartilage, decreased in serum and decreased production in immune cells;
- IL-8 is increased in synovial membrane and bone, decreased in serum;
- GM-CSF and IL-2 increased production in immune cells, decreased in bone;
- IL-1α, increased in synovial membrane, cartilage and bone, decreased in serum.

Similar variations in different tissues were recorded for:

- VEGFs, increased in synovial fluid and bone;
- -GF-ß isoforms, increased in cartilage and bone.

The collected data was insufficient for quantitative synthesis. Only two studies could be used for just three markers, and so no meta-analysis was performed.

	SF	SM			Cartilage				Serum	Bone							Immune Cells		Blood	
	Abrams, 2014 [10]	Hulejova, 2007 [7]	Takeshita, 2012 [23]	Saxler, 2005 [5]	Hulejova, 2007 [7]	Hashimoto, 2013 [14]	Verdier, 2003 [24]	Pombo-Suarez, 2009 [20]	Hulejova, 2007 [7]	Hulejova, 2007 [7]	Koorts, 2012 [16]	Shi, 2002 [22]	Kumarasinghe, 2012 [17]	Sanchez-Sabaté, 2009 [21]	Hopwood, 2007 [15]	Lavigne, 2004 [18]	Dallos, 2009 [11]	Granchi, 2003 [13]	Granchi 2006 [12]	Pape, 2000 [19]
BAFF																	↑↑*			
BMP-1														=[b]						
BMP-5															↓↓↓↓					
BMP-6														=[b]						
CGRP			↑↑↑*	↑↑↑↑*																
GM-CSF											↓↓							↑↑↑↑		
ICAM																↑↑				
ICAM-3															↑↑↑↑					
IFN-γ	↑↑										↓↓↓							↑↑↑↑		
IL-10		↑↑↑↑*			↑↑↑↑*				↓↓*	↑↑↑↑*	↓↓↓↓*							↓↓↓↓*		
Il-12											↓↓↓↓									
IL-1Rα	↓↓↓↓																			
IL-1α		↑↑↑↑			↑↑↑*				↓↓↓	↑↑↑↑*										
Il-1β	↓					=[b]					↓↓	=[b]								
IL-2											↓							↑↑↑↑*		
IL-4											↓							↓↓↓↓*		
IL-5											↓↓↓↓									
IL-6	↑↑↑↑										↑	↑*[b]			↑↑↑↑			↓		↑↑↑↑*
IL-8		↑↑↑↑*			↓	=[b]			↓	↑↑↑↑*	↑									
MCP-1	↑																			
MIP-1β	↑↑↑↑																			
NF-Kb			↑↑↑*																	
OPG																			↑↑↑*	
PDGF-ββ	↓↓↓↓																			
PGE-2												↑*[b]								
RANKL																			↓↓	
RANTES	↓↓↓																			
SP				↑↑↑↑*																
TGF-β											↑↑↑*		=[b]							
TGF-β1							↑↑↑	↑↑↑*/↑↑* [a]							↑↑↑↑					
TGF-β2							=	↑↑↑* [a]						↑*[b]						
TGF-β3							↑↑	↑↑↑*/↑↑* [a]						↑*[b]						

	↑/=				=[b]						
TGF-βR1											
TNF-α	↓↓↓↓	↑↑↑↑	↑↑↑*	↑↑↑	↑↑*	↑↑↑↑*	↓		↓↓↓↓		↑↑
TωI-1			↑↑*								
VEGF	↑↑↑										
VEGF-b								↑↑↑↑			
VEGF-c								↑↑↑↑			

Table 2. Ratios on neuroimmune expression between hip osteoarthritis and controls according to the sample

Ratios were computed as times raised: ↑: 1–1.25; ↑↑: 1.25–1.50;↑↑↑: 1.50–2; ↑↑↑↑: >2; and times decreased: ↓: 1–0.8; ↓↓: 0.8–0.67; ↓↓↓: 0.67–0.5; ↓↓↓↓: <0.5. When values are not available a single arrow (when statistically significant) or = (when non significant) was displayed. Data on statistically significance weren't available or statistically significant, unless indicated as *p < 0.05 SF synovial fluid, SM synovial membrane, Blood Whole Blood, hOA hip Osteoarthritis, C Controls. [a]PCR and Immunohistochemestry. [b]Values not available

	Sinovial Fluid	Synovial Membrane	Cartilage	Serum	Immune Cells	Blood	Bone
Increased	IFN-γ IL-6 MCP-1 MIP-1β VEGF	IL-10 IL-1α IL-8 TNF- α TGF-β1 TGF-β2 TGF-β3 CGRP NF-Kb TuJ-1 SP	IL-10 IL-1α TNF- α TGF-β1 TGF-β2 TGF-β3	TNF- α	BAFF GM-CSF IFN-γ IL-2	IL-6 OPG TNF- α	BMP-1 BMP-6 ICAM ICAM-3 IL-6 IL-8 PGE-2 TGF-β1 TGF-β2 TGF-β3
Doubtful or Equal			IL-8 Il-1β				Il-1β TGF-β TGF-βR1 TNF- α
Decreased	IL-1Rα Il-1β PDGF-ββ RANTES TNF-α			IL-8	IL-10 IL-4 IL-6 TNF- α	IL-10 IL-1α RANKL	BMP-5 GM-CSF IFN-γ IL-10 IL-12 IL-1α IL-2 IL-4 IL-5 VEGF-b VEGF-c

Table 3. General pattern of neuroimmune expression
Analysis presented was based on articles general results. When 2 articles had conflicting data on expression or when comparisons were stated as non-significant data was assigned as doubtful or equal

Discussion

Recent studies showed the joint-specific character signature of the immunity and nervous system activity underlying OA (27). This is the first systematic review on the neuroimmune expression of patients with hip OA. Few articles were available, and even fewer when sorted among samples studied. Most of the literature regarding hip OA is focused on the immune response and pathological changes of immune mediators. On neurochemical expression, only two articles that meet our inclusion criteria were retrieved. Both showed a tendency to neuropeptide overexpression in synovial membrane (5, 25).

Although this review did not found any specific systematic pattern in each individual tissue, some tendencies on the general neuroimmune expression were observed. Pro-inflammatory cytokines such as IL-6, TNF-α and IL-8 were found local and/or systemically increased in the context of hip OA. Particularly, IL-6 is locally increased in synovial fluid and bone, and also systemically in blood. IL-6 is a pro-inflammatory cytokine, that acts as a stimulator of osteoclast recruitment and bone reabsorption, being related with altered bone metabolism previously described in OA (28, 29). This goes along with previous works postulating OA as a pro-inflammatory condition (1, 2), and is reinforced by the significant raise of other pro-inflammatory cytokines, such as TNF-α and IL-8. TNF-α was found augmented, both systemic and locally, in synovial membrane, cartilage, bone and blood. It acts both as a mediator of matrix degradation (30) and as an intermediate between immune and nervous system. It is associated with nociceptive response and induces neuronal ingrowth (25, 31). IL-8 was found increased in both bone and synovial membrane, presenting a pattern of expression similar to IL-1.

IL-10 and IL-4 are known anti-inflammatory cytokines. Previous works reported that they are spontaneously produced in synovial membrane and cartilage (30), probably in an attempt to locally control the inflammatory process (9, 30). This review supports the findings on IL-10, which is increased in both tissues, but no information was retrieved on IL-4 expression in hip OA patients. Also, the systemic decrease of these anti-inflammatory markers in serum and immune cells, reinforce the ongoing idea of a shift towards a pro-inflammatory state, already reported in hip OA (1, 2)

A local response on cartilage and bone was also observed when analyzing TGF-ß family cytokines. TGF-ß is an inductor of chondrocyte anabolic response and is antagonized by IL-1, that acts as a stimulator of cartilage degradation (18, 32). Accordingly, both TGF-ß1, - ß2 and -ß3 and IL-1 isoforms were found increased in these tissues. However, no data on the systemic expression of TGF-ß was retrieved, and although one article reported a systemic decrease of IL-1 expression (9), these results were not significant, supporting the theory of a tendency to a local action of IL-1, with no measurable systemic repercussion (33, 34).

RANKL is an osteoclastogenic factor that triggers a cascade of intracellular events, essential to osteoclast activation and differentiation. OPG is a RANKL decoy receptor and limits its biologic activity. Therefore, OPG activation suppresses osteoclast differentiation, inhibits their activation and induces apoptosis (14). Granchi et al described an increased expression of OPG in hip OA patients, stating that elevated OPG levels may reflect a protective mechanism of the skeleton to compensate for the osteolytic activity that

occurs in severe osteoarthritis (14). However, in this article, RANKL expression comparisons were not statistically significant (14).

The two articles retrieved on neurochemical expression in patients with hip OA, reported a raise of CGRP, Tuj-1 (neuron-specific class III ß-tubulin) and SP in synovial membrane (5, 25).

The role of nerve fibers and their neurotransmitters in cartilage, subchondral bone, and other joint tissue function and homeostasis is becoming more evident, with reports on the peripheral nervous system involvement in the pathogenesis of disorders such as OA. Suri et al. reported the presence of both sensory (SP- and CGRP-positive) and sympathetic nerve fibers (neuropeptide Y (NPY)-positive) in the articular cartilage, within vascular channels, in both mild and severe stages of knee OA. The exclusively perivascular localization of nerves in the surface layer of articular cartilage implies vascularization as a driving force behind its innervation (35). Nerve growth is associated with peripheral sensitization. Accordingly, the presence of nerves in structures such as cartilage that are not normally innervated could expose them to chemical stimulation and mechanical stress, explaining why perivascular nerve growth might contribute to the pain mechanisms in OA (8), and particularly in hip OA (5).

Tuj-1 is a neuron-specific class III ß-tubulin that was found in the synovial membrane of patients with hip OA, being absent in the normal controls (31). The expression of this neurochemical marker occurs after blood vessels and nerve fibres ingrowth from the inflammation of synovial tissue. These inflammatory mechanisms are probably associated with the pain complaints of patients in hip OA (36).

Clinical data from OA patients supports an association between CGRP-immunoreactive fibers and pain (37). This review retrieved two articles showing an increased expression of this neuronal marker in patients with hip OA, what may also be associated with the pain mechanisms in this condition.

Additionally, in other diseases, such as hip dysplasia, increased levels of SP and CGRP were detected in synovial tissue and fluid and were associated with catabolic and pro-inflammatory effects (38). SP, also found increased in hip OA patients, was implicated in the modulation of the physiological metabolism of chondrocytes and cartilage homeostasis, with catabolic effects on articular cartilage during OA (39).

This is corroborated by other works stating the importance of these peptides in modulation of the inflammatory process and in signaling of pain in OA (40), being increased in all stages of inflammation (41).

Overall, our review goes along with previous reports on OA, with no relevant differences found between hip OA neuroimmune expression and the one reported in general OA patients (24). A recent review on immune expression showed a tendency towards an overexpression of cytokines in patients with OA, with a role for inflammation in the disease severity and progression (4). Our review confirms these results, showing an overall increase of cytokine expression in OA and reinforcing the idea of a link between a deregulated function of the neuroimmune system and the development and perpetuation of the disease (4). Nevertheless, no specific systematic pattern on neurochemical changes in OA was found. This work brings light on the need to further studies on the neuroimmune axis in joint-related conditions, as its role is yet to be clearly defined.

The individual methods found in the retrieved works were heterogeneous. Some studies provided no definition for the control group, and others used patients with proximal femur fractures as controls. Even if stated by individual authors that no OA was observed when this last group was used as control, one cannot exclude both the influence of the fracture itself and the possible influence of concomitant milder undiagnosed forms of OA. Patients affected with proximal femur fractures are elderly subjects, which can be affected by milder forms of hip OA, with local and systemic biochemical changes before the time a radiological diagnosis of hip OA is made (42). Also, fractures, due to the inherent aggression, are associated with both a local and a systemic biochemical response, with an increased inflammatory response (43). Both undiagnosed hip OA and the fracture-associated inflammatory reaction can lead to an underestimation of the neuroimmune activation in patients with hip OA, which represents an import bias in these comparisons (42). Furthermore, it is known that expression of individual molecules changes along OA progression. Since many articles do not state the stage of the disease in which each sample collected, one cannot reliably assure that the comparisons established refer to similar timing of the disease, what can have a major impact in the results presented. Lastly, the revised studies used different methodologies to assess the targets across the analyzed tissues. Thus, the sensitivity (e.g. ELISA vs Lumina(R) Multiplex) and the evaluated form of the target (e.g. mRNA vs. protein) limits the reliability of a molecular hip OA profile (44).

Our study has some limitations. Firstly, a quantitative data synthesis by meta-analysis was not possible, as only a few studies were available in each

molecule expression for a specific tissue, with different outcome measurements. Also, most studies included a small number of patients, a problem also stated in a previous review, that implies a need for future confirmation of these data in additional studies or in larger cohorts (4). Some studies reported results only from qualitative outcomes, and others do not report the significance of their quantitative results. This further impacts our ability to properly analyze their results. As stated before, one study was even excluded since no reliable data was provided.

Nevertheless, this is the first available systematic review on neuroimmune expression in human patients with OA, and especially with hip OA, without any limitation for sample size, age group, sex or type of sample studied. Two blinded reviewers analyzed the articles in each review phase, diminishing the risk of selection bias. Only 4 full-texts were not available, with high full-text article retrieval rate.

Future studies with strictly defined rules on control and patient selection, as well as disease progression stage, demographic characteristics of samples, sample collection, processing and analysis are needed. As stated by previous reports, a correlation with clinical features of the disease may also be a valuable resource in future strategies for directing therapy investigations (4). Few information was available on neurochemical activity in these patients, and in what comes to immune system, most studies focus only on classic cytokines. New and more information on different and recent found targets are required (2, 11, 45). Also, and particularly for hip OA, there is a need to study the role of neuroimmune expression on the functional impairment and pain levels reported by these patients. It is also important to have a previously

defined set of molecules with central roles in this disease, to have a more uniform report among future works. Larger samples are needed to provide more reliable results.

Conclusions

This is the first systematic review available on neuroimmune expression on hip OA and highlights a key role of inflammation in both disease maintenance and progression. It is associated with an overall upregulation of the neuroimmune system, confirming previous reports on a deregulated balance between pro and anti-inflammatory cytokines, both locally and systemically, impacting cartilage and bone remodelling. This review enhances the importance of further studies with a simultaneous assessment on immune and neurochemical expression in these patients, following clearly defined criteria and similar methodological strategies.

Declarations

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Competing Interests

All authors declare no competing interests in the development and publication of this study.

Authors' contributions

MRS contributed to study conception and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision and final read and approval upon submission. DL contributed to study conception and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision and final read and approval upon submission. DV contributed to study conception, analysis and interpretation of data, drafting of

the manuscript, critical revision and final read and approval upon submission. JA contributed to interpretation of data, drafting of the manuscript and final read and approval upon submission. NN contributed to interpretation of data, drafting of the manuscript and final read and approval upon submission. GC contributed to study conception, interpretation of data, drafting of the manuscript and final read and approval upon submission. ML contributed to study conception and design, interpretation of data, drafting of the manuscript, critical revision and final read and approval upon submission.

Ethics

Not applicable.

Consent to publish

Not applicable.

Additional Files

Additional File 1

Technique	Sample	Art.	Group	BAAF	BMP-1	BMP-5	BMP-6	CGRP	GM-CSF	ICAM	ICAM-3	IFN-γ	IL-10	IL-12	IL-1Rα	IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	MCP-1	MIP-1β	NF-Kb	OPG	PDGF-ββ	PGE-2	RANKL	RANTES	SP	TGF-β	TGF-β1	TGF-β2	TGF-β3	TGF-βR1	TNF-α	TuJ-1	VEGF	VEGF-b	VEGF-c
Bead-based multiplex immunoassay (µg/mL)	SF	Abrams, 2014 [10]	hOA									199.12 (188.9)	1.5 (0.6)		1108.07 (1032.1)	0.4 (0.1)	11.36 (11)*				182.5 (93.4)	2.4 (2.8)	44.04 (25)	26 (13)			56.87 (22)			188.08 (49.4)						79.22 (70.6)		258.17 (60.2)		
			C									151.06 (123.2)	0.2 (0.02)		4026.03 (3183.3)	0.2 (0.04)	13.12 (11.9)*				61.3 (30.4)	0.4 (0.3)	35.93 (12.1)	9 (4)			270.71 (151)			341.95 (85.5)						198.25 (140.7)		140.52 (38.2)		
	SM	Hulejova, 2007* [7]	hOA																																	1.9 (0.5)				
			C																																	0.8 (0.1)				
	C	Hulejova, 2007 [7]	hOA																			13.5 (4.1)														2.5 (0.5)				
			C																			16.4 (9.2)														1.5 (0.3)				
	S	Hulejova, 2007 [7]	hOA																			9.2 (1.4)														11.8 (4)				
			C																			9.4 (1.1)														8.8 (0.7)				
	Bo	Hulejova, 2007* [7]	hOA																			11.7 (5)														2.3 (0.6)				
			C																			1.1 (0.5)														0.4 (0.01)				
		Koorts, 2012 [16]	hOA						1.96 (7.4)*			0.1 (0)*	2.17 (3.5)*	0.63 (0.6)*			0.37 (0.9)*	4.07 (10)*	1.24 (3.2)*	1.11 (5.8)*	4.37 (3.4)*	16.38 (9.6)*										16.69 (6.2)*					2.05 (1.5)*				
			C						2.58 (10.6)*			0.18 (0.4)*	4.56 (3.9)*	2.73 (5)*			0.49 (1)*	5.04 (11.9)*	1.48 (2.3)*	2.23 (4.4)*	3.56 (5.6)*	14.2 (19.4)*										8.6 (6.9)*					2.29 (1.1)*				
ELISA (pg/mL) - mean (sd)	SM	Hulejova, 2007* [7]	hOA																																						
			C																																						
	C	Hulejova, 2007 [7]	hOA																																						
			C																																						
	IC	Dailos, 2009* [11]	hOA	685.0 (574.8-809.3)*																																					
			C	527.3 (462.1-568.0)*																																					
		Granchi, 2003* [13]	hOA						2335 (869)			1925 (849)	86 (64)					1692 (400)	0.1 (0.001)		3008 (894)															850 (206)				
			C						963 (145)			861 (266)	434 (141)					498 (105)	73 (18)		3027 (535)															1949 (972)				
	BI	Granchi, 2006 [12]	hOA																							4044 (1932)			1115 (1074)											
			C																							2586 (1082)			1606 (1245)											
		Pape, 2000 [19]	hOA																		46 (16)															0.1 (0.05)				
			C																		12 (18)															0.07 (0.06)				
	S	Hulejova, 2007 [7]	hOA										17.5 (1.9)			4.1 (0.5)																								
			C										22.0 (2.1)			7.3 (0.6)																								
	Bo	Hulejova, 2007* [7]	hOA										1.2 (0.6)			0.2 (0.05)																								
			C										0.1 (0.01)			0.05 (0.01)																								

		Shi, 2002[%] [12]	hOA														NI				NI							NI													
			C														NI				NI							NI													
		Kumarasinghe, 2012[%] [17]	hOA																														NI									
			C																														NI									
qRT-PCR (fold-change)	C	Pombo-Suarez, 2009 [20]																																1.6	1.7	2.3						
		Hashimoto, 2013 [14]															NI					NI																			
	Bo	Sanchez-Sabaté, 2009 [21]			NI		NI																												NI	NI	NI					
Microarray (fold-change)	Bo	Hopwood, 2007 [15]				-10.31					3.30										2.59												2.74			-4.92				3.24	5.30
Immunostaining (median range) (all imunohistochemestry except article 12 - immunocitology)	SM	Takeshita, 2012 [23]	hOA					54%																	68%												58%	46%			
			C					0%																	0%												0%	0%			
		Saxler, 2007 [5]	hOA					15.6 (14.3-18.8)[¥]																							8.2 (6.1-9.1)[¥]										
			C					5.7 (3.9-7.2)[¥]																							3.2 (1.8-4.2)[¥]										
	Bo	Lavigne, 2004[%] [18]	hOA							48.6%																															
			C							37.5%																															
	C	Pombo-Suarez, 2009 [20]	hOA																															+++	++	++						
			C																															++	+	+						
		Verdier, 2007 [24]	hOA																															+++	±	+++	++					
			C																															+/++	±	++	+/++					

Table S1. Comparison of neuroimmune expression between hip OA and controls in the included studies

Values are grouped by assessment technique and tissue studied. Articles are presented as references for space purposes. All articles were in vivo, except for the ones marked. Data is presented with 2 decimal cases, as Mean (Standard Deviation), otherwise indicated. *Median (25^{th}-75^{th}). [#]ng/g. [&]pg/mL. [¥]fibers/cm^2. [%]In vitro. [$]Bead based multiplex immunoassay. Art.:Article. SF: synovial fluid. SM: synovial membrane. C: Cartilage. Bo: Bone. Bl: Whole Blood. S: Serum. IC: Immune Cells. hOA: hip Osteoarthritis. C: Controls. NI: No information.

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5-6
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	4-5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4-5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	4
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	4-5; Figure 1
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5-6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5-6
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	N/A
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	6

Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	6
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	N/A
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	6
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	6-7; Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	7-8; Table 1
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	N/A
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	8-9; Table 2
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	Table 3
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	N/A
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16)).	Figure 2
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	9
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	13
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	10-14
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	15

Table S2. PRISMA Checklist
N/A: Non applicable. *From:* Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed100009

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CHAPTER III

IMMUNE RESPONSE AND INNERVATION SIGNATURES IN ASEPTIC HIP IMPLANT LOOSENING

Article 2

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Immune response and innervation signatures in aseptic hip implant loosening

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Abstract

Aseptic loosening (AL) of hip prosthesis presents inflammation and pain as sign and symptom similarly to arthritis pathologies. Still, the immune and innervation profiles in hip AL remain unclear and their interplay is poorly explored. Herein, local tissue inflammatory response, sensory and sympathetic innervation as well as associated local mediators were assessed in hip joint microenvironment underlying AL and compared to osteoarthritis (OA).

Histopathological analysis, immune cells (macrophages, T, B cells and PMNs) as well as sensory and sympathetic nerve fibers (Substance P+, CGRP+, TH+) distribution and profiles were analyzed on tissues retrieved from patients with failed hip prostheses due to AL (n=20) and hip OA (n=15) by immunohistochemistry. Additionally, transcriptional levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-12a, iNOS), anti-inflammatory cytokine (IL-10), osteoclastic factor (RANKL) and bone remodeling factor (TGF-β1) were locally evaluated by qRT-PCR. Serum TGF-β1 levels were assessed preoperatively by ELISA.

Histopathological analysis revealed that tissues, aseptic interface membranes of AL patients had distinct tissue architecture and immune cells profile when compared to OA synovial tissues. Macrophages, T cells and B cells showed significant differences in tissue distribution. In OA, inflammation is mostly confined to the vicinity of synovial membrane while in AL macrophages infiltrated throughout the tissue. This differential immune profile is also accompanied with a distinct pattern of sensory and sympathetic innervation. Importantly, in AL patients, a lack of sympathetic innervation aseptic interface

membranes without compensation mechanisms at cellular levels was observed with simultaneous reorganization of sensorial innervation. Despite the different histopathological portrait, AL and OA patients exhibited similar transcriptional levels of genes encoding key proteins in local immune response. Nevertheless, in both pathologies, TGF-β1 expression was prominent in sites where the inflammation is occurring. However, at systemic level no differences were found.

These findings indicate that AL patients exhibit different local inflammatory response and innervation signatures from OA patients in hip joint. These insights shed the light on neuro-immune interplay in AL and highlight the need to better understand this crosstalk to unravel potential mechanisms for targeted-therapies to improve hip joint lifetime and treatment.

Keywords: osteoarthritis, aseptic loosening, prosthetic debris, immune response, hip innervation

Introduction

Osteoarthritis (OA) has long been considered a cartilage driven “wear and tear” disease that may lead to hip joint failure (1, 2). The pain and diminished hip joint motion induced by OA may be effectively treated through primary hip replacement (3, 4). Unfortunately, hip replacement is not a permanent solution as prostheses often fail 15-25 years after primary surgery, mostly due to aseptic loosening (AL), infection and dislocation (5, 6). Other therapeutic solutions are then urgently required to expand implants’ lifetime.

Inflammation is common to AL and OA (1, 7, 8). Immune cells and cytokines have been previously reported in both clinical scenarios (8-11). Synovial inflammation is often observed in OA patients and is frequently characterized by the infiltration of specific immune cell populations, such as macrophages, T cells and mast cells, as well as by the expression of pro-inflammatory cytokines, as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (7). Nevertheless, these findings were mostly described in studies focusing on the knee or in studies combining data from both knee and hip, without anatomical discrimination (7). On the other hand, periprosthetic joint inflammation is commonly known as a complex local biological response that takes place on synovial membrane-like interface tissues, triggered by implant released by-products (particles and ions) (12-15). Of note, the nature, size and amount of released particles are recognized to define the inflammatory profile (13-16). While polymeric particles such as polyethylene (PE) and polymethylmethacrylate (PMMA) are often associated with macrophage mediated foreign body reaction and tissue fibrosis, high levels of metallic

particles and ions have been demonstrated to promote tissue necrosis and lymphocyte-driven responses (17).

Pain is one of the clinical features observed in both AL and OA conditions (18, 19). Innervation profile is mostly studied in the context of arthritic diseases but not in the presence of implantable biomaterials. Patients and animal studies highlighted innervation of synovial tissues as a possible player in the pain process and, based on anatomical mapping, suggested a coupling of innervation and inflammation in osteoarthritic synovial tissues (20, 21). It is well documented that both sympathetic, tyrosine-hydroxylase (TH)+ or neuropeptide Y (NPY)+, and sensory nerve fibers, substance P+ and calcitonin gene related peptide (CGRP)+, are present in OA synovial tissue and grow towards cartilage along blood vessels (21). On the other hand, so far, two studies addressed the innervation of the interface membranes surrounding AL hip prostheses (19, 22). Unfortunately, the data is still unclear. Niissalo et al reported that the synovial membrane-like interface did not contain C-sensory peptidergic or sympathetic neural structures while Ahmed et al identified sympathetic nerve fibers in these interface membranes (19, 22). Thus, innervation profile and its possible association with periprosthetic joint inflammation, triggered by prosthetic debris in AL scenario, should be revised. Furthermore, the comparison of the inflammatory versus innervation profiles of AL and OA has never been examined and requires further investigation. In this study, the immune and innervation profiles of AL and OA were addressed dissecting local players at tissue, cellular and molecular levels with potential systemic translation.

Materials and Methods

Patients and Samples

Centro Hospitalar São João ethics committee approved this study and all patients consented to the use of their tissue and blood for research purposes. The followed procedures were in accordance with the Helsinki Declaration of 1975, as revised in 2000. Samples from synovial membrane-like interface tissue/aseptic interface membrane were collected from twenty patients during hip revision surgeries due to AL of hip prostheses. All revised hips had a metal-on-polyethylene (MoP) coupling, eleven out twenty cemented and bone defects were classified according to the Paprosky classification (23). Relevant clinical information is summarized in Table 1. Infection, recurrent dislocation and periprosthetic fracture were considered exclusion criteria in the aseptic loosening group. Osteoarthritic synovial tissues were collected from fifteen patients undergoing primary hip replacement surgeries for primary OA. OA patients were classified for OA severity according Tönnis OA grade (24) and presented scores from moderate (2) to severe (3). The clinical information of these patients is summarized in Table 2. For both OA and AL groups, the same orthopedic team performed the collection of tissue samples. Previously to surgery, blood was collected and leukograms and plain radiographies were registered.

Immediately after excision, both OA synovial tissues and aseptic interface tissues were split. Half of the tissue was immersed in formalin for further histological analysis, while dry ice was used to freeze the remaining tissue until storage at -80ºC. No more than 4 hours elapsed between tissue collection and storage.

Aseptic Loosening Case Number	Age	Gender	Hip prostheses type	Implant Fixation	Time to Revision (months)	Component Revised	Type of bone defect	Metallosis
1	79	M	MoP	Cemented	13	Acetabular	2B	
2	61	F	MoP	Cemented	24	Acetabular	2A	
3	74	F	MoP	Cemented	23	Acetabular	2B	
4	86	F	MoP	Cemented	46	Acetabular	2B	
5	50	F	MoP	Uncemented	56	Acetabular	3A	Yes
6	79	F	MoP	Cemented	96	Acetabular	3A	
7	73	M	MoP	Cemented	108	Acetabular	3A	
8	69	F	MoP	Cemented	117	Acetabular	2C	
9	74	M	MoP	Cemented	120	Acetabular	2A	
10	77	M	MoP	Uncemented	120	Acetabular	2B	
11	45	F	MoP	Uncemented	129	Acetabular	3A	
12	73	F	MoP	Uncemented	130	Acetabular	1	Yes
13	63	F	MoP	Cemented	138	Acetabular	3A	
14	75	M	MoP	Cemented	144	Acetabular	3A	
15	62	F	MoP	Uncemented	153	Acetabular	2C	
16	53	F	MoP	Uncemented	156	Acetabular	2A	
17	86	F	MoP	Cemented	168	Acetabular	2A	
18	71	F	MoP	Uncemented	204	Acetabular	1	
19	82	F	MoP	Uncemented	216	Acetabular	1	Yes
20	75	M	MoP	Uncemented	240	Acetabular	1	Yes

Table 1. Clinical data from aseptic loosening patients

Osteoarthritis Case Number	Age	Gender	OA severity (Tonnis Classification)
1	79	F	2
2	45	F	3
3	54	F	3
4	80	F	3
5	55	M	3
6	49	F	2
7	76	M	3
8	51	F	2
9	56	F	3
10	71	M	3
11	74	F	3
12	83	M	3
13	37	F	3
14	74	M	3
15	66	M	2

Table 2. Clinical data from osteoarthritis patients

Histochemistry and immunostaining

Half of collected tissues were formaldehyde-fixed paraffin embedded and cross-sections of 3 µm thickness were cut. Contiguous sections were stained with hematoxylin & eosin (H&E) and Masson's trichrome (MT). For immunohistochemistry, tissue sections were deparaffinized and rehydrated before heat induced antigen retrieval (98ºC, 10 mM citrate buffer, pH 6.0). Endogenous peroxidases were blocked using 3% H2O2 and non-specific binding sites were blocked using Background Block (Cell Marque, USA), previously to the incubation with antibody diluent 1% BSA (negative control) or with primary antibodies: anti-CD3 (clone PS1, dilution 1:100, Biocare Medical, USA), anti-CD20 (clone L26, dilution 1:100, Cell Marque, USA), anti-CD68 (clone 514H12, dilution 1:100, Novocastra, UK), anti-CD163 (clone MRQ-26, dilution 1:150, Cell Marque, USA), anti-HLA-DR (clone TAL1B5, dilution 1:5000, Abcam, USA) and anti-TGF-β1 (clone TB21, dilution 1:2000, Abcam, USA). After primary antibody incubation, tissue sections were incubated with BrightVision Poly-HRP-Anti Mouse/Rabbit/Rat IgG (Immunologic, the Netherlands) and then revealed using DAB Plus Substrate System (Thermo Scientific, USA), before hematoxylin counterstaining. The specificity of immunostainings of CD20, CD163, CD68, HLA-DR and CD3 was confirmed using human spleen as positive control.

For immunofluorescence studies, tissue sections were deparaffinized and antigen retrieval of rehydrated sections was performed using Proteinase K (0.2 mg/mL in PBS) for NF200 immunostaining and incubated for 20 min at 98ºC in Citrate buffer (pH 6.0), or TE Buffer (pH 9.0) for Substance P and CGRP staining, respectively. After quenching endogenous fluorescence with

0.1 % sodium borohydride and 100 mM NH4Cl, sections were incubated with blocking buffer (10 % FBS, 1 % BSA, 0.2 % Triton X-100). Primary antibody rabbit anti-human neurofilament heavy subunit (NF200) (dilution 1:1000, Abcam, USA), anti-Substance P (dilution 1: 1000, Millipore, USA), anti-TH (dilution 1:100, Millipore, USA), anti-CGRP (dilution 1:4000, Sigma-Aldrich, USA) or blocking buffer (negative control) was applied overnight at 4 ºC. For signal detection, tissue sections were incubated with anti-rabbit Alexa Fluor 568 antibody (dilution 1:1000, Life Technologies, USA), incubated with DAPI and then mounted with Fluoroshield Mounting Medium (Abcam, USA). The specificity of immunostainings for NF200, Substance P, TH and CGRP was confirmed using a specimen of human Morton's neuroma as positive control.

Semi-quantitative histopathological evaluation

In order to characterize synovial microenvironment, tissues were semi-quantified considering the total immunoreactive area and scored into four categories: absent (0), present (1), frequent (2) or abundant (3), according to the histological grading system illustrated in the supplementary figures Figure S1 (tissue reaction), Figure S2 (prosthetic debris accumulation) and Figure S3 (immune cells distribution), similarly to the methodology followed by others (25, 26).

Structural changes, fibrosis and necrosis in OA synovial tissues and aseptic interface tissues were evaluated after H&E and MT staining. The thickening/hyperplasia and increased villi of synovial membrane were assessed in OA patients. The accumulation of polymeric, ceramic and metallic particles was assessed in synovial membrane-like interface tissues. A

polarized filter was used to detect polymeric particles. Ceramic and metallic particles were studied through Scanning Electron Microscopy (SEM) and their elemental composition assessed by Energy Dispersive Spectroscopy (EDS). Additionally, a phase contrast filter (Ph3) was used with an optical microscope to ease the detection of clusters of ceramic or metallic particles in histological slices, allowing a deep study of the interaction between particles and cells. The infiltration of target immune cell populations, identified by immunohistochemistry, was determined regarding the presence of macrophages (CD68+ cells), T cells (CD3+ cells) and B cells (CD20+ cells). The semi-quantification of polymorphonucleated cells (PMNs) was performed after PMNs identification as these cells have a lobed nucleus while multinucleated giant cells (GC) were detected due to their bigger size, multiples nucleus and CD68+ labeling.

Tissue samples retrieved from five out thirty-five patients (4/15 OA and 1/20 AL) were excluded from histopathological evaluation because they do not correspond to OA synovial tissue or aseptic interface tissue. H&E, MT and immunohistochemistry slices were analyzed using a light microscope Olympus CX31 while immunofluorescence, polarized light and phase contrast Ph3 images were captured on Carls Zeiss Axiovert 200 inverted microscope.

Gene expression analysis

Synovial tissues were homogenized in liquid nitrogen using a mortar and pestle to preserve RNA integrity. RNA was extracted and purified using TRIzol (Invitrogen, UK) and Direct-zol™ RNA MiniPrep (ZYMO Research, USA), according to the manufacturers' instructions. The amount and quality of

extracted RNA were evaluated by Nanodrop ND-1000 (Thermo Fisher Scientific, USA) and running RNA samples in a 2% agarose gel. The transcriptional levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-12a and iNOS), anti-inflammatory cytokine (IL-10), osteoclastic factor (RANKL) and bone remodeling factor (TGF-β1) were evaluated by quantitative real time PCR (qRT-PCR) in PCR iQ™5 system (Bio-rad, USA). All used primers, listed in Table 3, were optimized and melting curves of PCR products were evaluated to guarantee primers specificity. β2 microglobulin (B2M) and β-actin were used as reference genes. Experiments were performed in triplicated. Relative gene expression levels were calculated using the quantification cycle (Cq) method, according to MIQE guidelines (27). Fourteen out thirty-six cases (5/15 OA and 9/20 aseptic loosening) were excluded from gene expression analysis when average Cq for reference genes was above 26, to avoid bias in the evaluation of genes with later expression.

Gene	GenBank number	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
B2M	[NM_004048]	CCAGCGTACTCCAAAGATTCAG	AGTCAACTTCAATGTCGGATGG
β-actin	[NM_001101]	TACCTCATGAAGATCCTCA	TTCGTGGATGCCACAGGAC
IL-1β	[NM_000576]	CTTCAGCCAATCTTCATT	CACTGTAATAAGCCATCAT
IL-6	[NM_000600]	CAATCTGGATTCAATGAGGAGACT	CTGTTCTGGAGGTACTCTAGGTAT
IL-10	[NM_000572]	GGAGAACCTGAAGACCCTCA	TATAGAGTCGCCACCCTGAT
IL-12a	[NM_000882]	TACCAGGTGGAGTTCAAG	GTTCTTCAAGGGAGGATTT
iNOS	[NM_000625]	AATTGAATGAGGAGCAGGTC	TCCTTCTTCGCCTCGTAA
TNF-α	[NM_000594]	TCTCTCTAATCAGCCCTCTG	TGCTACAACATGGGCTACAG
RANKL	[NM_003701]	GGATGGCTCATGGTTAGA	CAAGAGGACAGACTCACTT
TGF-β1	[NM_000660]	CCTGGACACCAACTATTG	CTTGCGGAAGTCAATGTA
Y1R	[NM_000909]	AAGAGGATTGTTCAGTTCA	GATTGGTTTGGTTGTTATAGA
Y2R	[NM_000910]	ACTCTTACCTATACCTTAATG	GTGATTGTGGATACTTGT
Y5R	[NM_006174]	AAGGAAGGGAAAGGGTGTTAC	CGAGTGGCAGCAGTATTATTCT
VMAT2	[NM_003054]	TGCGGGATTCTGCATCATGT	CATCCAAGAGTACCAGGGCG

Table 3. List of primers used for quantitative qRT-PCR analysis

ELISA

Serum transforming growth factor β1 (TGF-β1) concentrations were measured using Quantikine® ELISA Kit for human TGF-β1 (R&D Systems, USA), according to the manufacturer's protocol. Cytokine concentration was calculated against a standard curve.

Statistical analysis

Statistical analysis was performed using SPSS 21.0 (SPSS Inc. Chicago, IL, USA). The level of significance was set at $p < 0.05$ (*). Visual histogram analysis and Kolmogorov-Smirnov test were used to evaluate the normal distribution of continuous variables (gene expression data, TGF-β1 plasma concentration and percentage of immune cells in blood). Accordingly, these variables were analyzed using Student's t-test or its non-parametric counterpart, Mann-Whitney test. Tissue immune response and innervation was histologically classified in different grading categories and comparisons between aseptic interface tissues and OA synovial were done using Chi-square test. All graphs were prepared using Prism software (GraphPad software, San Diego, CA, USA).

Results

Local immune responses in OA synovial tissues and synovial membrane-like interface tissue

In this study, fifteen patients underwent primary hip replacement due to OA (Figure 1A) and twenty hip implants were revised and their acetabular defects classified by X-ray imaging (Figure 1B). Macroscopically, the collected synovial tissues at primary and revision surgeries were morphologically distinct. Synovial surface was white and with diffuse papillary architecture (dashed black line, Figure 1C and D) whereas synovial membrane-like interface tissues were highly fibrotic (Figure 1E) or with greyish appearance (Figure 1F), in case of metallosis.

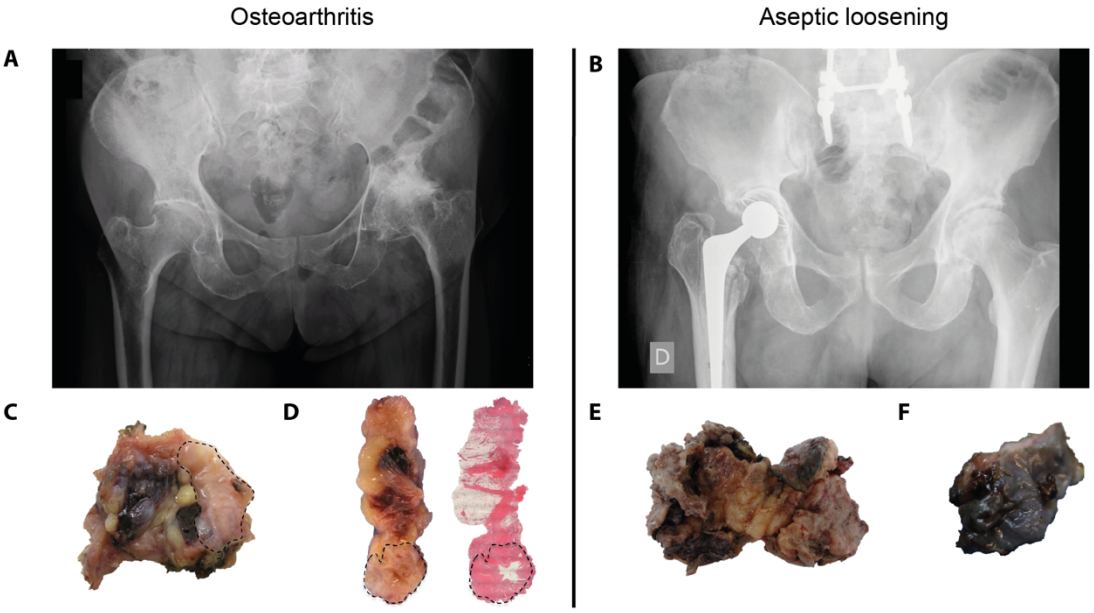

Figure 1. Radiological and macroscopic tissue features of OA, AL and metallosis.
(A) Anteroposterior X-rays showing OA of the hip. (B) Failed metal-on-polyethylene total hip joint due to AL. (C,D) Macroscopic images of OA synovial tissue collected at primary hip replacement. (E) Synovial membrane-like interface tissue retrieved at hip revision surgery due to AL. (F) Metallic debris accumulation in synovial membrane-like tissue from AL patient with metallosis.

Semi-quantitative analysis of tissue organization and immune cell distribution in OA synovial tissue and aseptic interface tissues showed distinct local profiles. OA synovial membranes displayed a bicellular lining layer (LL) and a sublining layer (SLL) (Figure 2A) enriched in blood vessels (black arrows) and collagen (Figure 2B), as well as structural changes induced by synovial inflammation, such as synovial hyperplasia (Figure 2C and D) and increased number of villi (Figure 2E and F). OA patients presented at least one sign of synovial inflammation (thickening or villi) but their magnitude was variable among patients (Figure 2C and E). Synovial membrane-like interface tissues presented fibrotic stroma with increased collagen deposition (Figure 2G and H) than OA synovial tissues ($p=0.001$) and with some necrotic regions (Figure 2I).

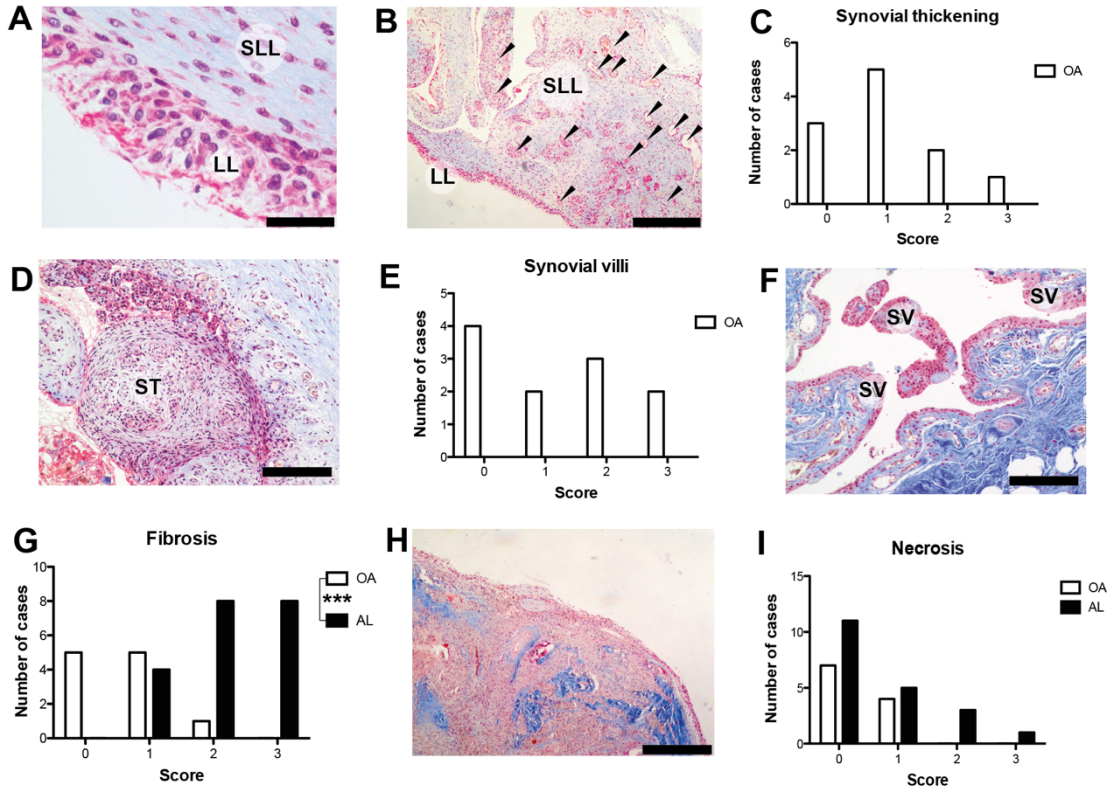

Figure 2. Histological evaluation of tissues organization
(A) OA synovial tissue is composed by different cell types and matrixes organized in synovial lining layer (LL) and sublining layer (SLL). **(B)** Dense net of blood vessels (black arrows) was found in SLL together with loose connective tissue (blue stain). **(C)** Histological grading for synovial membrane thickening in OA. **(D)** Synovial membrane thickening (ST) with reactive neo vascularization. **(E)** Histological grading for synovial villous expansion in OA. **(F)** OA synovial membrane presenting villous hypertrophy (SV). **(G)** Histological grading for tissue fibrosis. **(H)** Synovial membrane-like interface tissue showing fibrosis characterized by intense deposition of collagen fibers (blue stain). **(I)** Histological grading for tissue necrosis. Masson's trichrome (A,B,D,F,H) staining. Scale bars correspond to 500 µm (B,F,H), 200 µm (D) and 50 µm (A). Semi-quantitative histological evaluation of tissue architecture was performed in specimens retrieved from 11/15 OA and 19/20 AL patients. *** $p<0.001$. Chi-square test was used to compare OA and AL groups.

The accumulation of prosthetic debris in synovial membrane-like interface tissues was semi-quantified. Polymeric particles were just detected in 11/20 analyzed aseptic interface tissues (Figure 3A) after tissue analysis under polarized light (Figure 3B and C). No significant difference was found between AL patients with cemented and uncemented MoP bearings regarding the amount of polymeric particles entrapped in synovial membrane-like tissues. Zirconia particles (ZrO_2) were observed in almost all synovial membrane-like interface tissues retrieved from patients with loose cemented prostheses (Figure 3D-F) and mainly phagocytized by macrophages or multinucleated giant cells (Figure 3G). Ph3 contrast filter eased the detection of ZrO_2 particles (Figure 3E and F). White color particles under Ph3 filter were confirmed to be ZrO_2 and to be organized in clusters of nanoparticles by SEM/EDS (Figure 3H and I). Intense deposition of metallic particles was just found in the four cases of metallosis that were patients with uncemented metal-back acetabular cups (Figure 3J). Aseptic interface membranes with metallosis presented high deposition of metallic particles with concomitant macrophage infiltration (Figure 3K), tissue fibrosis (Figure 3L) and necrosis (Figure 3M). Under Ph3 filter, metallic nanoparticles and haemoglobin presented violet and red color respectively, which allow distinguishing them from ZrO_2 particles (Figure 3N and O).

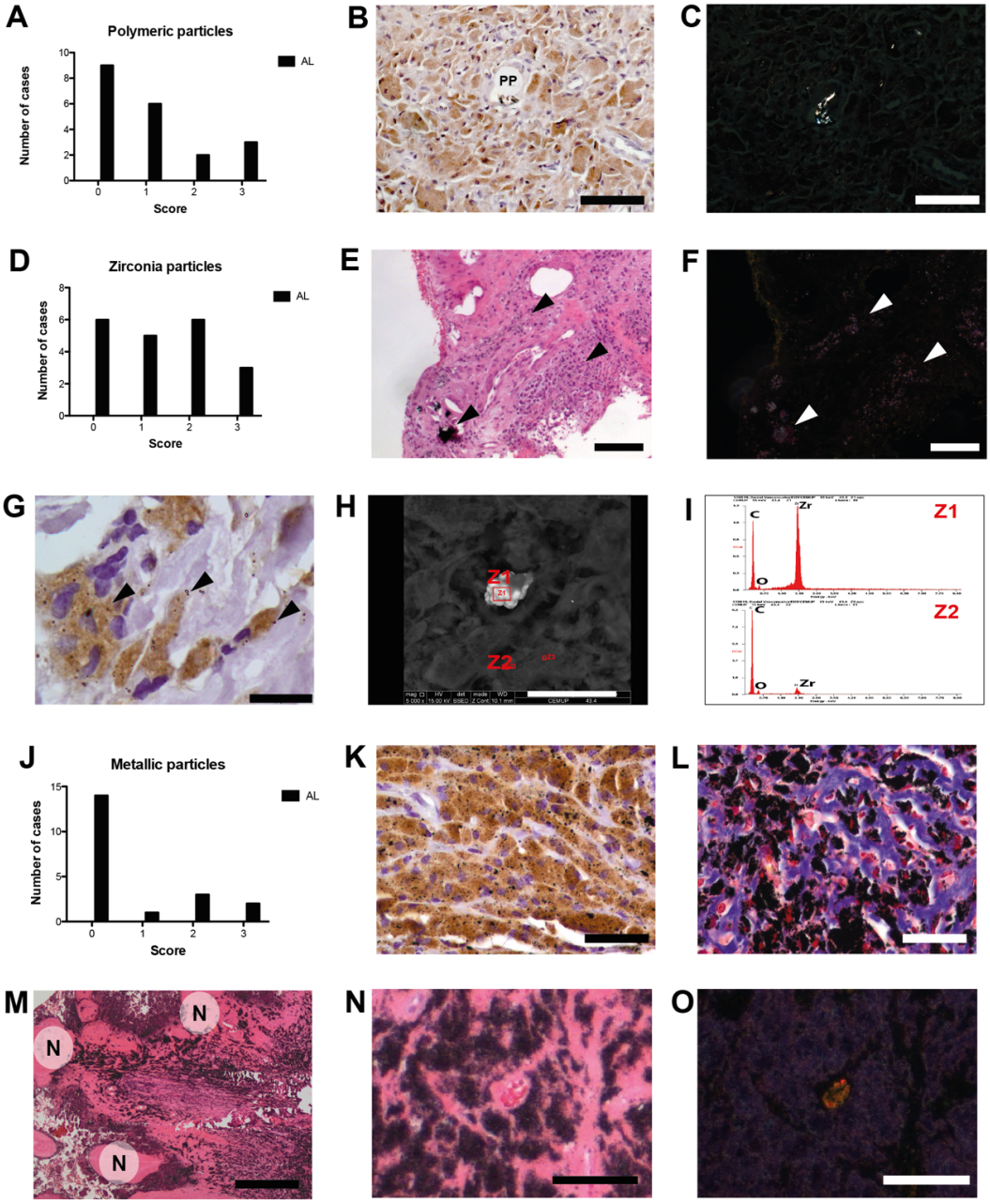

Figure 3. Prosthetic debris accumulation in synovial membrane-like tissues
(A) Histological grading for accumulation of polymeric particles (PP) in aseptic interface membranes. **(B)** PP surrounded by macrophages (brown cells). **(C)** Same section of (H) under polarized light showing birefringent PP. **(D)** Histological grading for deposition of ZrO_2 particles in synovial membrane-like interface tissues. **(E)** H&E image of aseptic interface membrane with intense deposition of ZrO_2 particles (black arrows) using conventional light microscopy. **(F)** Same tissue region observed using Ph3 filter with white and bright particles corresponding to ZrO_2 debris (white arrows). **(G)** Macrophages (brown cells) phagocytizing ZrO_2 debris (black arrows). **(H)** SEM image of synovial membrane-like interface tissues containing clusters (Z1) and sole (Z2) ZrO_2 nanoparticles. **(I)** EDS analysis confirming the elemental composition of ZrO_2 nanoparticles. **(J)** Histological grading for entrapment of metallic particles in tissues. **(K)** Macrophages (brown cells, CD68+ cells) with phagocytized metallic particles. **(L)** Metallic particles co-localized with macrophages and high deposition of collagen (blue stain). **(M)** Necrosis (N) in regions of aseptic interface membranes with massive accumulation of metallic particles. **(N)** Tissue from an AL patient with metallosis showing metallic particles and erythrocytes. **(O)** Same tissue section shwoing bright metallic particles and erythrocytes but exhibited under Ph3 filter violet and red colors, respectively. H&E staining (E,M,N), polarized light (C), Ph3 filter (F,O), Masson's trichrome (L) immunohistochemistry (B,G,K), SEM imaging (H) and EDS spectra (I). Scale bars correspond to 500 µm (E,M), 50 µm (B,K,L) and 20 µm (G,N,O). Semi-quantitative histological evaluation was performed in tissues retrieved from 19/20 AL patients.

Immune cell distribution was studied in tissues from AL and OA patients. In AL, macrophages ($CD68^+$ cells) were more abundant than in AL ($p=0.007$; Figure 4A), often confined to lining layer in OA patients (Figure 4B), while significant macrophage infiltration was found in synovial membrane-like interface tissues (Figure 4C). In these tissues, co-localization between polymeric particles and macrophages was often found (Figure 4D) and a similar pattern was observed in AL patients with cemented and uncemented MoP bearings. In AL patients, macrophages were the most prevalent immune cell population, even in metallosis cases, and in overall highly express M1 (HLA-DR; Figure 4E) and M2 (CD163; Figure 4F) markers. Multinucleated giant cells in foreign body reaction setting were mostly found in synovial membrane-like interface tissues ($p=0.037$, Figure 4G) surrounding big polymeric particles, likely PMMA, with ZrO_2 particles entrapped inside (Figure 4H). T cells ($CD3^+$ cells) (Figure 4J), and B cells ($CD20^+$ cells; Figure 4L) in lower number, could be detected in the majority of tissues collected from AL and OA patients (Figure 4I and K), including AL patients with uncemented implants or signs of metallosis. Moreover, the number of PMNs was also low in both aseptic interface membranes and OA synovial tissues (Figure 4M and N). Overall, the immune cell populations addressed, namely macrophages, T cells, B cells and PMNs, in this study were most located in the vicinity of synovial membrane in OA patients, while in AL patients these cells were identified throughout the aseptic interface membranes.

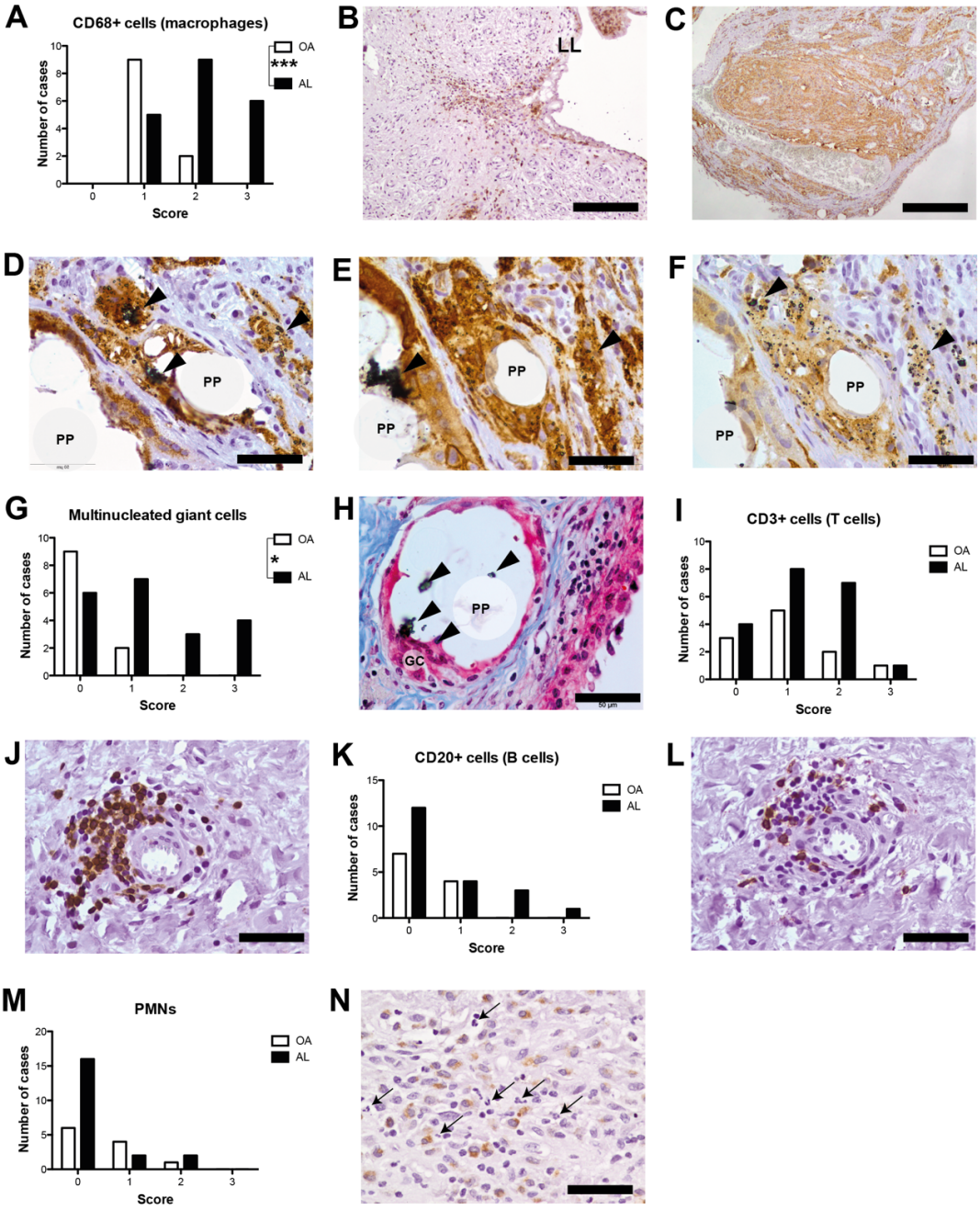

Figure 4. Immune cells distribution in OA synovial tissues and aseptic interface membranes
(A) Histological grading for macrophage (CD68+ cells) infiltration in tissues. **(B)** In OA, macrophages (brown cells) were almost found at LL. **(C)** Intense macrophage (brown cells) infiltration was detected in synovial membrane-like interface tissues. **(D)** In aseptic interface tissues, macrophages (brown cells) were often found surrounding or phagocytizing prosthetic debris such as polymeric particles (PP) and ZrO_2 particles (black arrows). **(E)** HLA-DR+ cells – an M1 macrophage marker. **(F)** CD163+ cells – an M2 macrophage marker. **(G)** Histological grading for multinucleated giant cells in tissues **(H)** Multinucleated giant cells (GC) phagocyting a big PP particle with ZrO_2 particles (black arrows) entrapped inside. **(I)** Histological grading for T cells (CD3+ cells). **(J)** Perivascular T cells (brown cells) clusters in OA synovial tissue. **(K)** Histological grading for B cells (CD20+ cells). **(L)** B cells (brown cells) in lymphocyte aggregates around blood vessels but in lower number than T cells. **(M)** Histological grading for polymorphonucleated cells (PMNs). **(N)** Increased number of PMNs (black arrows) in synovial membrane-like tissue with macrophages (brown cells, CD68+ cells). Masson's trichrome staining (H) and immunohistochemistry (B,C,D,E,F,J,L,N). Scale bars correspond to 500 µm (B,C) and 50 µm (D,E,F,H,J,L,N). Semi-quantitative histological evaluation was performed in synovial tissues retrieved from 11/15 OA and 19/20 AL patients. * $p<0.05$, *** $p<0.001$. Chi-square test was used to compare OA and AL groups.

Local innervation in AL and OA patients

Myelinated nerve fibers identified by NF200 immunoreactivity were detected in both synovial membrane-like interface tissues and OA synovial tissues. They were presented as single fibers (Figure 5A and C) and were preferentially arranged around blood vessels (Figure 5C) particularly in tissue regions of reactive vascularization induced by immune responses underlying AL and OA. Alternatively, nerve fibers were also found in neurome-like structures (Figure 5B and D) in both AL and OA patients. Sensory and sympathetic innervation, as seen by immunohistochemical markers of sensory nerve-associated peptides (Substance P and CGRP) and catecholaminergic marker of sympathetic neurons (TH), showed different pattern between OA patients and AL patients. TH immunoreactive nerve fibers were observed in OA synovial tissues (Figure 5F) but not in synovial membrane-like interface tissues (Figure 5G and H). Substance P and CGRP were found both in OA synovial tissues and synovial membrane-like interface tissues but showed different pattern. In OA synovial tissues, nerve fibers immunoreactive to Substance P and SGRP were observed mainly around blood vessels in the vicinity of synovial membrane (Figure 5J and N). In addition, a high number of cells in the OA synovial membrane also stained for TH, Substance P and CGRP (Figure 5E, I and M). In synovial membrane-like interface tissues, the expression of Substance P and CGRP was also found in both nerve fibers and cells but with a broad distribution throughout the tissue (Figure 5K, L, O and P).

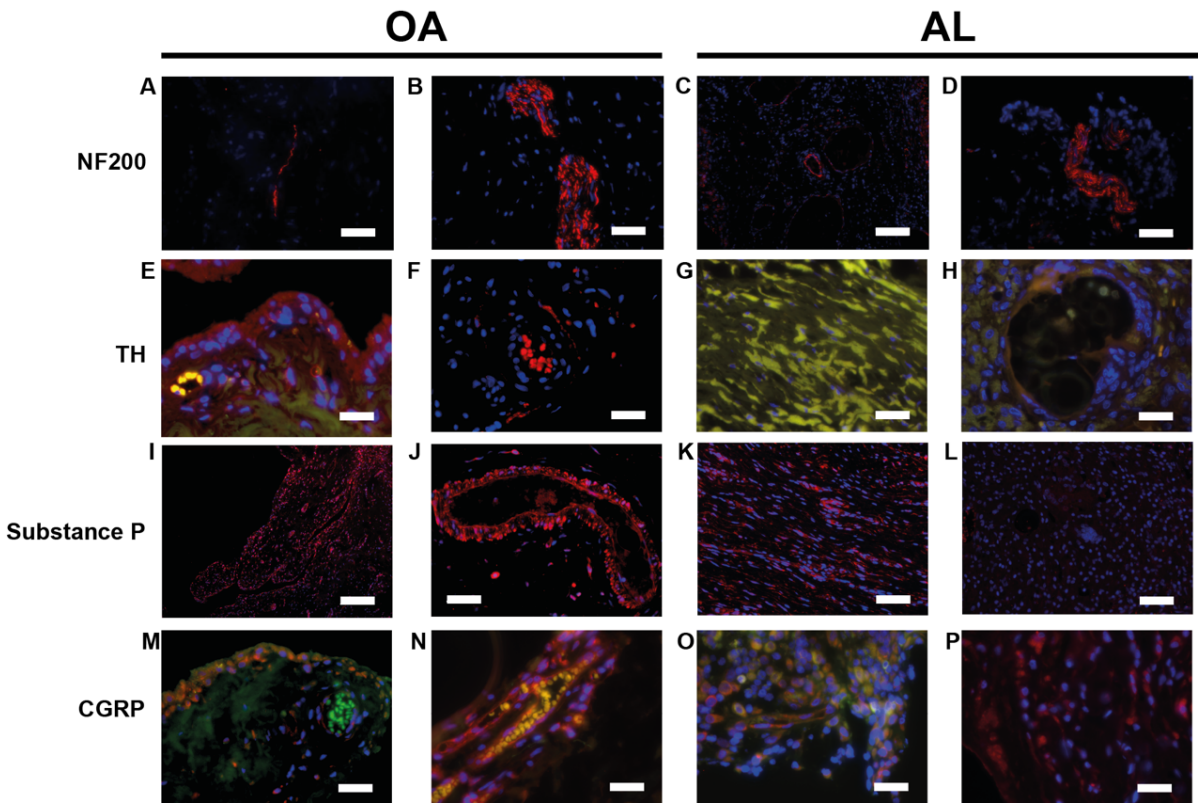

Figure 5. Local tissue innervation in OA and AL patients
(A-D) NF200+ fibers (red) as sole fiber or organized in neurome-like structures in OA synovial tissues as well as in aseptic interface membranes. **(E)** Synovial cells expressing TH (red). **(F)** TH+ fiber surround a blood vessel. **(G,H)** Nor fibrotic nor reactive tisue with giant cells presented positive labelling for TH. **(I)** Substance P+ cells in OA synovium membrane. **(J)** Substance P+ fibers and cells surrounding a blood vessel located at subintimia of OA synovial tissue. **(K,L)** In AL patients, Substance P+ fibers were just found in fibrotic regions. **(M)** CGRP+ cells in synovial membrane. **(N)** CGRP+ fibers along a blood vessel in OA synovial tissue. **(O,P)** Some cells expressing CGRP in aspetic interface tissue. red=NF200+ or TH+ or Substance P+ or CGRP+, blue=cell nuclei, green=autofluorescence) Scale bars correspond to 50 μm (A, B, D, F, G, H, I, J, K, L, M, N, O, P) and 200 μm (E). 100 μm (C).

Local gene expression profile

The expression levels of pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 were similar in aseptic interface tissues and OA synovial tissues (Figure 6A-C). Inducible nitric oxide synthase (iNOS) and IL-12a expression levels were found to be low when compared with the TNF-α, IL-1β and IL-6 mRNA levels detected in AL and OA groups (Figure 6D and E). Interestingly, the anti-inflammatory cytokine IL-10 presented a tendency (p=0.084) to be higher expressed in synovial membrane-like tissues than in OA synovial tissues (Figure 6F). Two genes involved in bone remodeling were evaluated: TGF-β1 and receptor activator of nuclear factor kappa-B ligand (RANKL). The mRNA levels of TGF-β1 were significantly reduced (p=0.038) in aseptic interface tissues when compared to OA synovial tissues (Figure 6G). However, no differences between AL and OA patients were verified for RANKL (Figure 6H) and no correlation was found between mRNA levels of RANKL and the bone defect type.

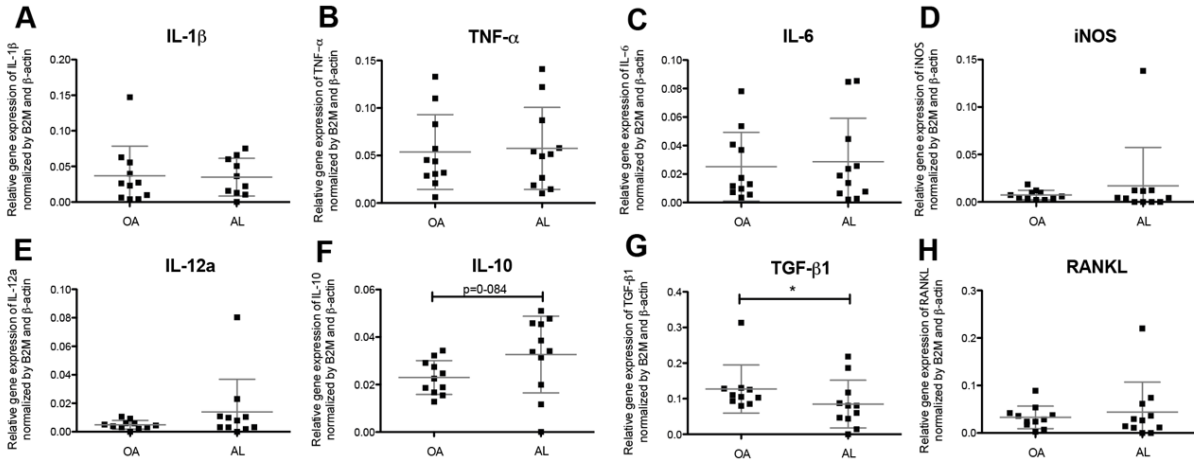

Figure 6. Gene expression profiles of cytokines in aseptic interface membrane and OA synovial tissues
Relative expression levels of genes of interest determined through qRT-PCR and normalized by two reference genes, β-actin and B2M: **(A)** IL-1β, **(B)** TNF-α, **(C)** IL-6, **(D)** iNOS, **(E)** IL-12a, **(F)** IL-10, **(G)** TGF-β1 and **(H)** RANKL. Data of 10/15 OA patients and 11/20 AL patients are shown. Significant difference of TGF-β1 expression between AL and OA groups (*p < 0.05). Mann-Whitney test was utilized to compare and analyze the obtainded data.

Local production of TGF-β1

In both AL and OA patients, TGF-β1 was detected in blood vessels endothelium cells (Figure 7A), macrophages (Figure 7B) and fibroblasts (Figure 7C). Interestingly, differences in the pattern of TGF-β1 expression were found between AL patients and OA. Aseptic interface tissues presented a trend ($p=0.1672$) toward increased number of regions expressing TGF-β1 (Figure 7D). In OA synovial tissues, TGF-β1 was present in the sublining layer of synovial membrane (Figure 7E), in aggregates of lymphocytes (Figure 7F) and blood vessels. In synovial membrane-like interface tissues, the distribution of TGF-β1-positive regions was heterogeneous (Figure 7G and H).

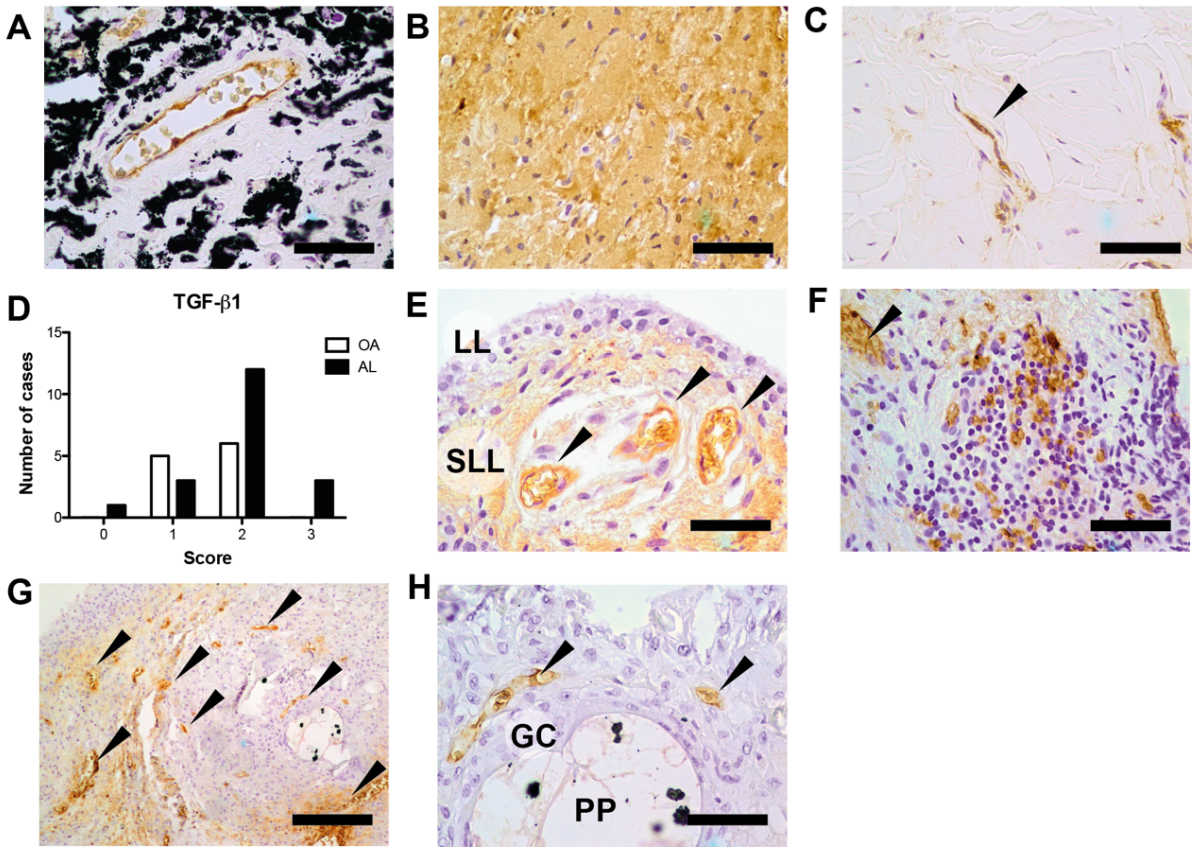

Figure 7. TGF-β1 expression in synovial membrane-like interface tissues and OA synovial tissues

(A) TGF-β1+ endothelial cells in aseptic interface tissue with metallosis. **(B)** TGF-β1+ macrophages in synovial membrane-like interface tissue. **(C)** TGF-β1+ fibroblast in aseptic interface membrane. **(D)** Number of OA and AL patients classified with score from 0 to 3 regarding the presence of TGF-β1 in tissue. **(E)** OA synovial membrane with positive labelling at sublining layer and endotethelium (black arrows). **(F)** Lymphocyte aggregate in OA synovial tissue with positive cells for TGF-β1. **(G)** Aseptic interface membrane presenting heterogenous TGF-β1 labelling. **(H)** Multinucleated giant cell (GC) phagocyting a polymeric particle (PP) with ZrO_2 particles inside (black clusters) with TGF-β1+ endothelial cells in the vicinity. Scale bars correspond to 500 µm (G) and 50 µm (A,B,C,E,F,H). Data was collected for 11/15 OA patients and for 19/20 AL patients. Chi-square test was used to compare OA and AL groups.

Immune cells proportions and TGF-β1 concentration in blood

The preoperative leukograms were analyzed and the concentration of TGF-β1 determined in plasma of both AL and OA patients. The percentage of circulating monocytes in both groups tended to be similar (Figure 8A). Interestingly, the percentage of lymphocytes seemed to be low in AL patients but within the reference interval in OA group (Figure 8B). Neutrophils did not present significant alterations compared with the reference values (Figure 8C). Remarkably, the levels of TGF-β1 in serum were similar in both groups (Figure 8D).

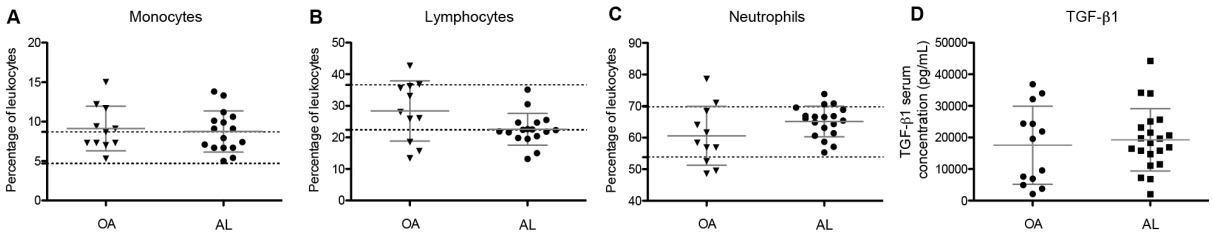

Figure 8 - Pre-operative evaluation of immune populations and TGF-β1 in blood
Data regarding the percentage of the following immune population in leukocytes count. **(A)** Monocytes. **(B)** Lymphocytes. **(C)** Neutrophils. Data was collected for 14/15 OA patients and for 17/20 AL patients. Dashed lines represent minimum and maximum reference values. **(D)** TGF-β1 serum concentration was determined for 13/15 OA patients and for all 20 AL patients.

Discussion

AL and OA differences rely on tissue architecture, immune cell distribution, local TGF-β1 expression as well as sensory and sympathetic synovial innervation. On the other hand, both pathologies share identical inflammatory mediators mRNA profiles and similar TGF-β1 concentrations in serum, as summarized in Figure 9.

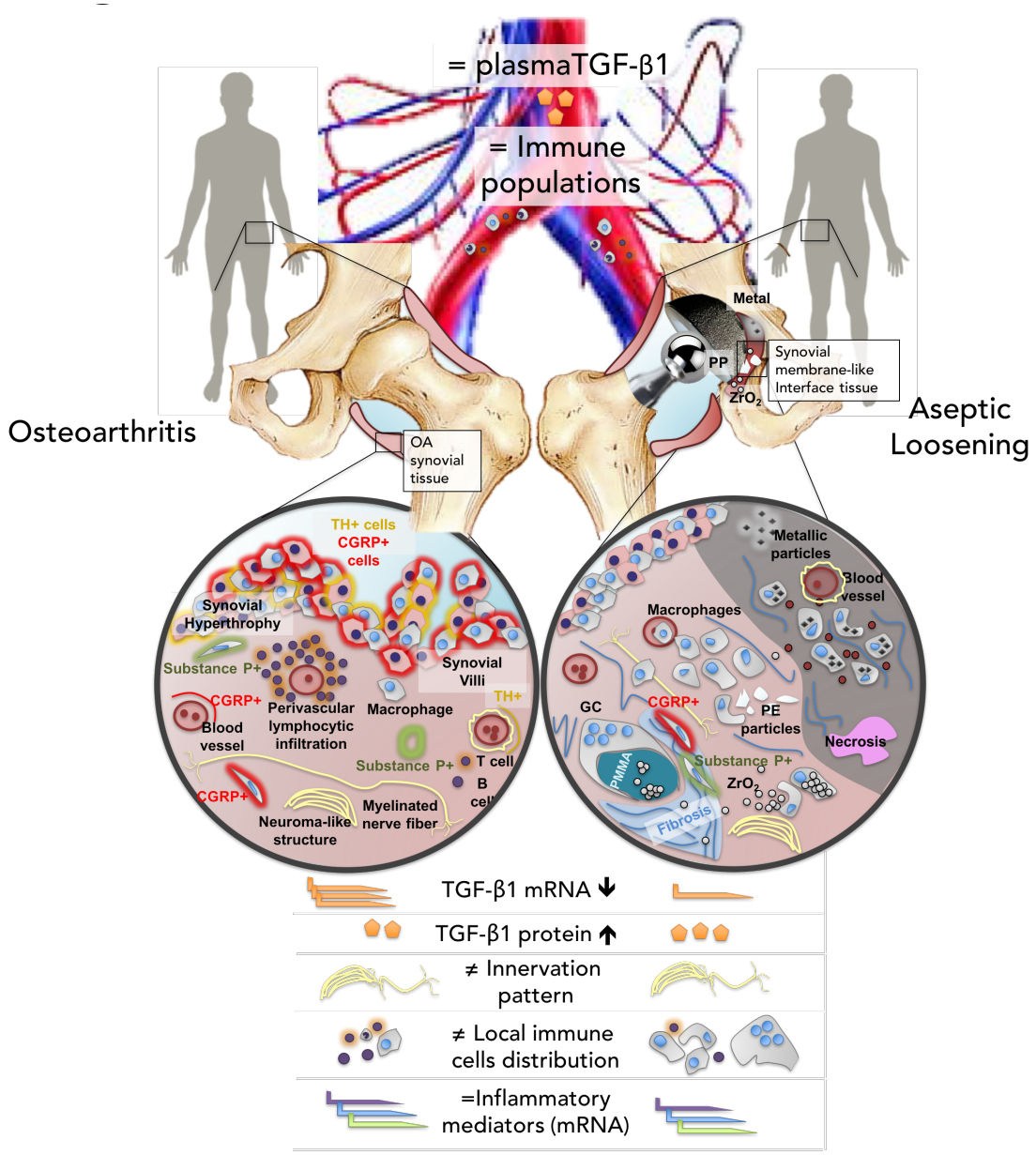

Figure 9. Overview of hip microenvironment in OA and AL

Synovial membrane-like interface tissues, present in the vicinity of loose prostheses, showed a macrophage-driven chronic inflammation. This immune response is likely influenced by both OA inflammatory background and the presence of prosthetic debris, leading to significant changes of local TGF-β1 expression but not systemically.

Distinct tissue organization and immune cell distribution were found in the tissues retrieved from the hip joint of AL and OA patients. OA synovial tissues presented signs of synovial inflammation (synovitis), such as synovial hyperplasia and villous hypertrophy, while aseptic interface membranes, formed after primary hip replacement, exhibited a particle-driven chronic inflammation. Intense infiltration of macrophages ($CD68^+$ cells) was observed in AL patients in comparison to OA synovial tissues. In synovial membrane-like interface tissues retrieved from AL patients, macrophages were the predominant immune cell type and were involved in the phagocytosis of small particles (< 10μm) or encapsulating bigger polymeric particles as multinucleated giant cells, in line with previous works about the role of macrophages on AL (28). Although PE particles, released by MoP prostheses, have been pointed out as AL catalyzers (29, 30), other types of particles may play a role on the pro-inflammatory microenvironment underlying osteolysis. Large amounts of metallic particles were observed in four out nine AL patients due to impingement of metallic components of uncemented MoP bearings.

Ceramic ZrO_2 particles, incorporated in bone cements for implants fixation as radiopacifier agent, were found in synovial membrane-like interface tissues from all the patients with cemented prostheses. Light microscopy with Ph3

contrast filter and SEM/EDS analysis revealed that ZrO_2 gradually migrate from cement particles (PMMA) to other regions of aseptic interface tissues in high number, namely after being phagocytized by macrophages. ZrO_2 particles were shown to be moderately toxic, activate macrophages, promote the expression of pro-inflammatory cytokines (TNF-α) and induce osteolysis *in vivo* (31, 32). Despite reported not toxic as metallic particles or studied as polymeric debris (12, 33, 34), ZrO_2 particles should not be neglected, as they are numerous, common and nano-sized with potential to unbalance inflammation towards osteolysis. It has been reported that particle-induced response is prone to drive macrophages towards M1 phenotype and that M1:M2 ratio was higher in synovial membrane-like interface tissue than in OA synovial tissues (35, 36). However, macrophage polarization in joint tissues remains controversial. In this study, the density of polymeric, metallic or ZrO_2 particles on aseptic interface tissues did not lead to local preferential macrophage polarization in M1 pro-inflammatory ($HLA\text{-}DR^+$) or M2 pro-regenerative phenotypes ($CD163^+$) as both receptors were similarly expressed on macrophages. In addition to the involvement of macrophages and lymphocytes in local inflammatory response, preoperative leukograms suggested monocyte expansion and contraction of lymphoid population in AL group.

Cytokines expression profile was found similar in synovial membrane-like interface tissues and OA synovial tissues, despite their distinct tissue architecture and immune cell distribution. The response of macrophages to prosthetic debris is believed to induce the production of the pro-inflammatory markers such as TNF-α, IL-1β, IL-6, IL-12a, and iNOS (28, 34, 37). However

those genes revealed similar mRNA levels in AL and OA patients, which is in line with comparable studies (26, 30, 38). The changes in cytokine expression induced by AL seem to be controversial (25). Reduced mRNA levels of IL-6 have also been described in aseptic interface membranes (26) while IL-6 was found significantly increase in synovial fluid of AL patients (25, 30). A trend for higher mRNA levels of the anti-inflammatory cytokine IL-10 was observed in synovial membrane-like interface tissues in comparison to OA synovial tissues ($p=0.084$). This result corroborates with other findings showing significant increase of IL10 protein levels in synovial fluid and interface tissues of AL patients (25, 26, 30). The up-regulation of IL-10 in aseptic interface tissues may constitute an attempt to balance the pro-inflammatory microenvironment induced by prosthetic debris (39, 40).

Although RANKL is involved in osteoclastogenesis and osteolysis, similar mRNA levels were found in AL and OA patients, in agreement with other authors (26, 30, 38, 41). In overall, identical pattern regarding the expression of cytokines was found in AL and OA patients. In the context of AL, no significant differences were registered between polyethylene-drive and metallosis cases as previously shown (42). The expression of TGF-β1 mRNA in synovial membrane-like interface tissues was found significantly lower than in OA synovial tissues but local TGF-β1 immunostaining suggested increased expression of this protein in AL patients in comparison to OA patients. These findings corroborate the results presented by other authors that have also found increased TGF-β1 mRNA levels in OA cartilage (9, 43, 44) and augmented TGF-β1 expression in synovial membrane-like interface tissues (45). In both AL and OA, TGF-$\beta1^{+}$ labeling was prominent in sites where the

inflammation is occurring and mostly expressed in macrophages, fibroblasts and endothelium cells. However, while in OA the TGF-β1^{+} cells were found in the region of synovial membrane, in AL, the distribution was more heterogeneous and throughout different tissue regions. These results suggest a possible association between TGF-β1 and the immune responses that underlie AL and OA. Despite a previous report indicates that in OA pathogenesis, TGF-β1 might have a pro-inflammatory effect by inducing fibroblasts to express TNF-α and IL-1β (46), its involvement in inflammatory response is not fully understood. Although the involvement of TGF-β1 in fibrosis is widely described, this growth factor may have dual effects on arthritic diseases (47). Both immune cells and fibroblasts are part of the complex microenvironments that underlie AL and OA and in which TGF-β1 may have different effects depending on levels of other inflammatory mediators. Additionally, in the context of particle-induced immune response, TGF-β1 has a conjoint role with other factors on bone remodeling (12).

Moreover, previous studies demonstrated that serum TGF-β1 has not predictive value to assess OA incidence and progression (48, 49). In our study, despite the difference in tissues regarding TGF-β1, AL and OA patients presented similar concentrations of TGF-β1 in serum.

The crosstalk between inflammation and innervation has been widely investigated in several joint-related disorders but not in presence of prostheses (50, 51). Previous studies have reported sensory and sympathetic innervation in joints diseases namely OA and rheumatoid arthritis (RA), where an inflammatory response is taken place within synovial tissues or in the vicinity of the articulation. Our study demonstrated for the first time distinct

pattern of sensory and sympathetic innervation in AL patients characterized by a loss of sympathetic nerve fibers when compared to OA patients. This differential profile has been also described in a comparative study showing lack of sympathetic innervation in RA patients while in OA patients this does not occur (52). The authors suggested that the reduction or absence of sympathetic innervation might be a consequence of the initial synovial inflammation, probably involving nerve repellent molecules (53), and a key factor to its maintenance in RA. In AL, a sustained chronic inflammatory response is maintained by the presence of prosthetic debris, primarily generated at the bearing surface of hip prostheses. Therefore, our findings, supported by those described in RA, strongly indicate a close correlation between sympathetic innervation pattern and the degree of the inflammatory response. A previous study described the appearance of TH^+ cells in the collagen-induced arthritis in mice and hypothesized that the presence of those cells might be a compensatory mechanism to the deprivation of sympathetic neurotransmitters in the joint (54). In AL patients, TH^+ catecholamine-producing cells were not detected in the synovial membrane-like interface, suggesting total uncoupling of local joint inflammation from the sympathetic activity (nerve fibers and cells).

Several animal and human studies have addressed the pattern of the sensory innervation in arthritis, namely in OA, and reported a significant re-organization of the sensory nerve fibers characterized by an alteration in the morphology, density and sprouting into areas of the joint that are normally poorly innervated (21). In this study, we showed that there is also alteration in the sensorial innervation of synovial membrane-like interface tissues in AL

patients with a notable distinct tissue distribution when compared to OA. Substance P and CGRP immunoreactive nerve fibers were observed in subintima regions (outer layer) mainly around blood vessels while in AL they were distributed throughout synovial membrane-like interface tissues. Of note, Substance P^+ and $CGRP^+$ cells were also found in these tissues as an additional source of these neuropeptides. Although, Substance P and CGRP are recognized as neuro-inflammatory modulators in arthritic joints, their functional role in the inflammatory response associated to prosthetic debris as well as in the reorganization of sensory and sympathetic nerve fibers in inflamed joint need to be clarified.

We acknowledge certain limitations in this study. To study the differences between AL and OA, thirty five patients were included in our study, an average number in the field (40). OA synovial tissues and synovial membrane-like interface tissues were retrieved by the same surgeon to minimize location bias. The histological semi-quantification of accumulation of prosthetic debris in aseptic interface tissues was limited to micro-sized particles, or clusters of nanoparticles, although tissues have been previously evaluated by SEM. Gene expression analysis was successfully performed in eleven out of twenty AL patients due to low RNA quality. Patients' stratification concerning implant fixation and metallosis (AL group) or disease severity (OA group) was performed but no significant effect was identified. On the other hand, the interpretation of the variability between patients may not be just influenced by a specific biological response in joint region but by other factors such as patients comorbidities (e.g. diabetes mellitus and dyslipidemia), geriatric condition, or genetic susceptibility to AL (16, 55).

Conclusion

Overall this study showed that AL and OA are two joint pathologies characterized by local immune response however with distinct tissue organization and immune cell distribution. This differential immune profile is also accompanied with changes of sensory and sympathetic innervation in hip joint. These findings highlight that the interplay between inflammation and innervation may be joint pathology-specific. Therefore, a deeper and conjoint understanding of these processes will constitute a solid base for targeted-therapies to improve hip joint lifetime and treatment.

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Additional Files

Additional File 1

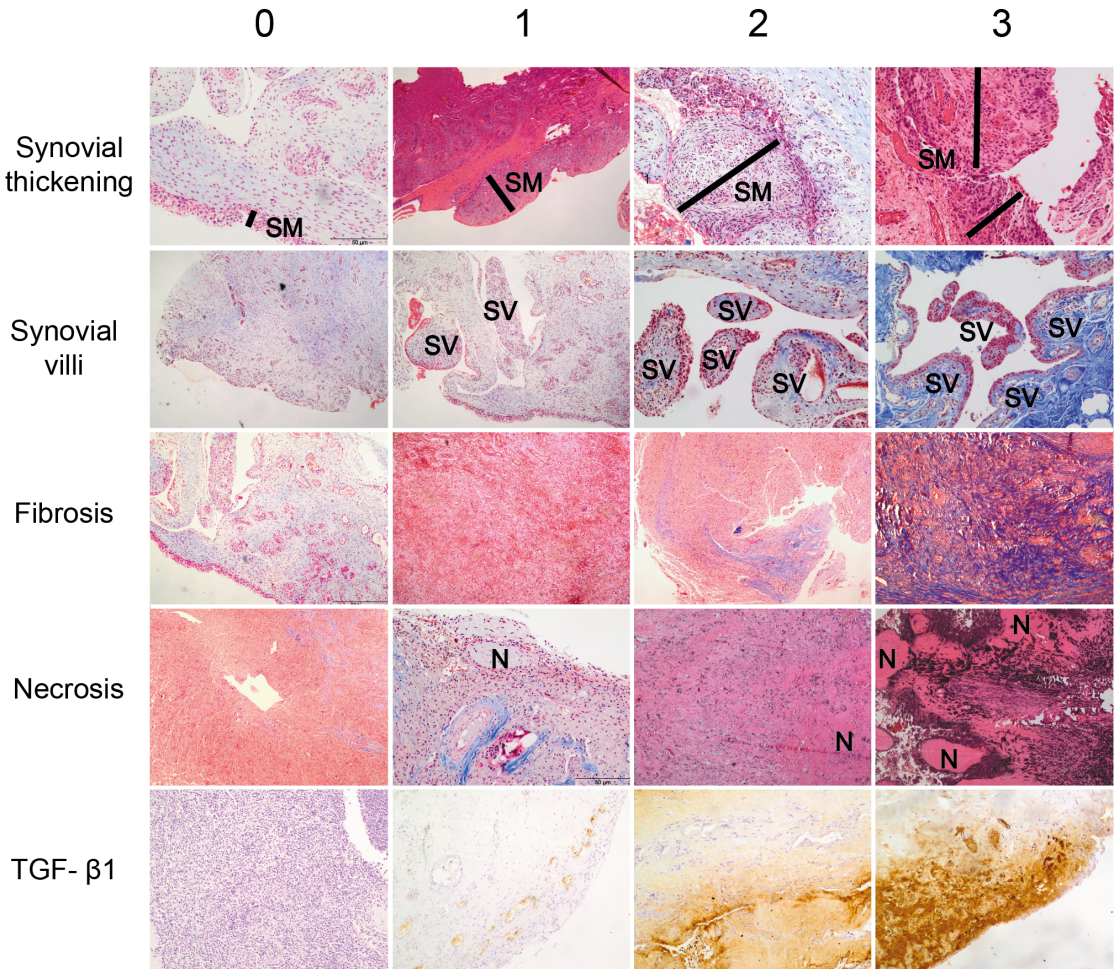

Figure S1. Histological grading applied in semi-quantification of OA synovial tissues inflammation and tissue fibrosis, necrosis, innervation (NF200) and TGF-β1 in tissues collected from OA and AL patients

Additional File 2

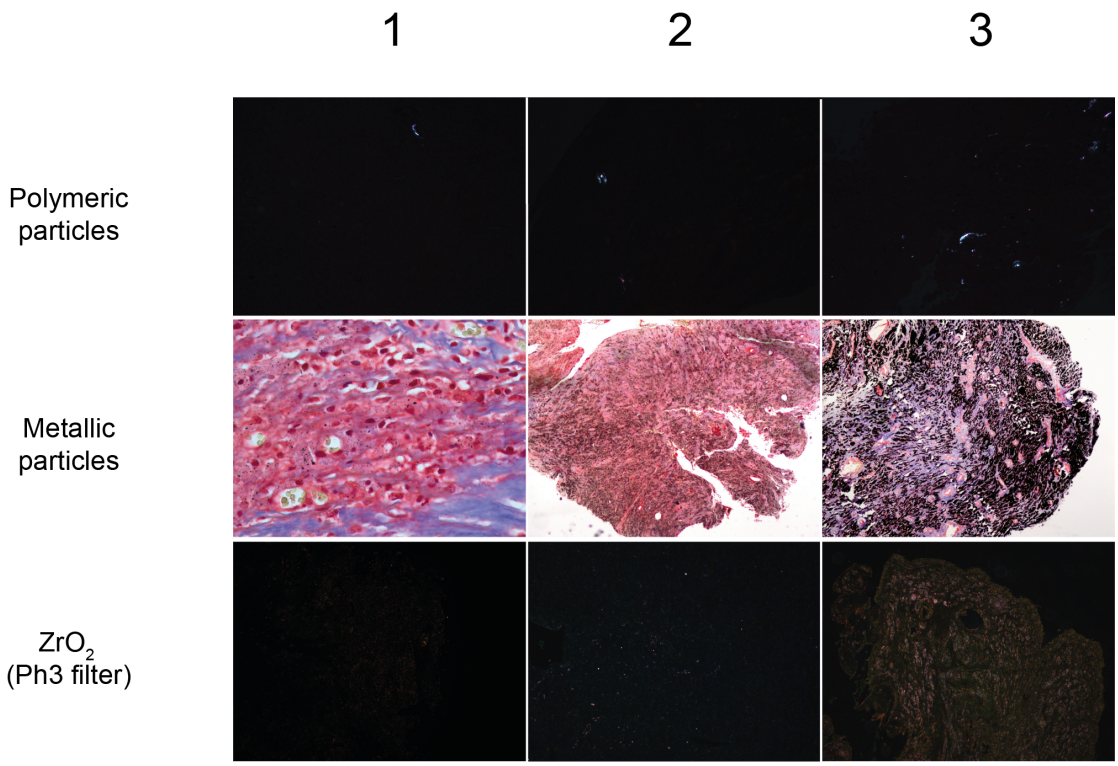

Figure S2. Histological grading applied in semi-quantification regarding the accumulation of prosthetic debris in synovial membrane-like interface tissues

Additional File 3

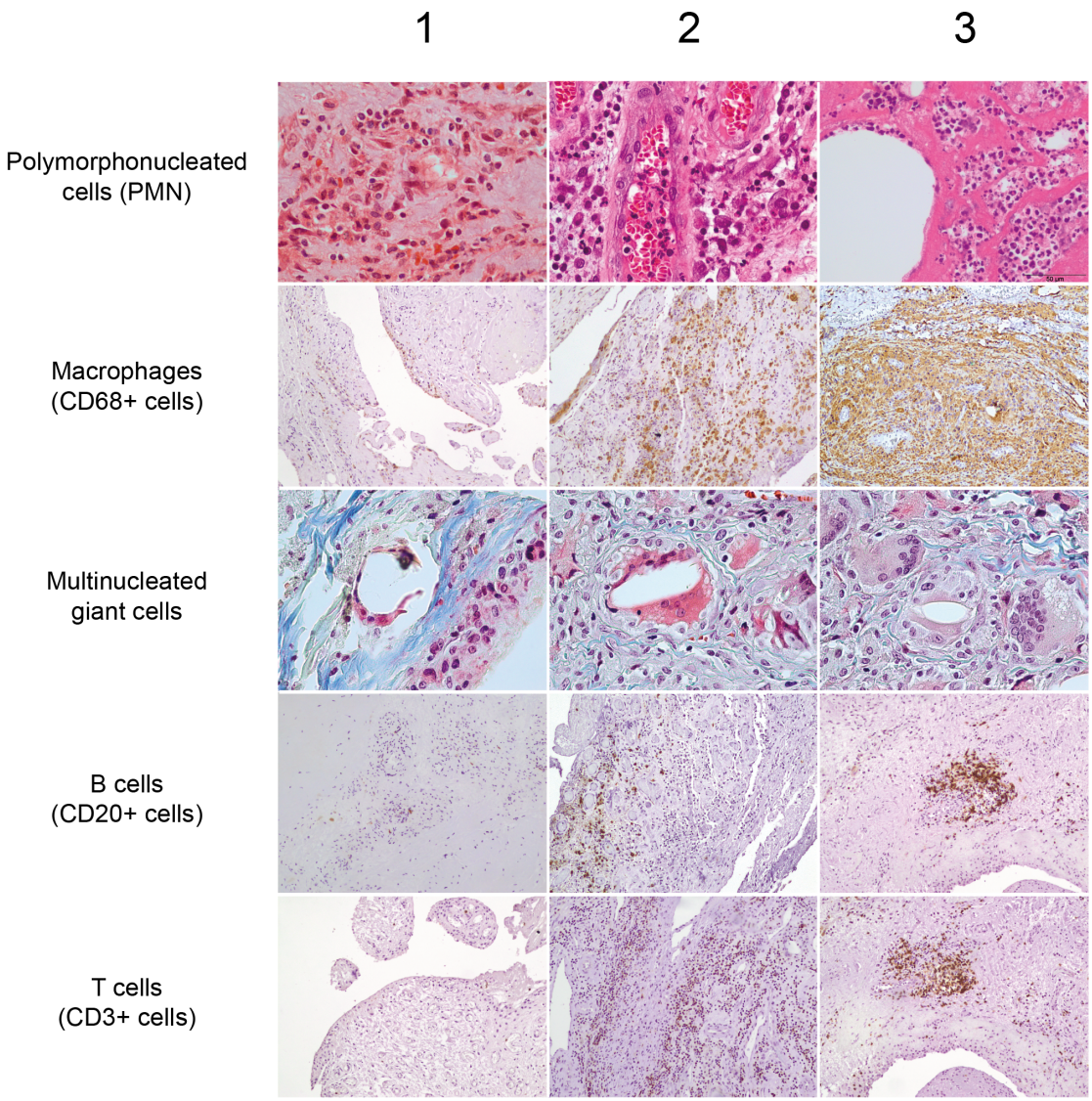

Figure S3. Histological grading applied in semi-quantification of immune cells prevalence and distribution in tissues retrieved from OA and AL patients

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CHAPTER IV

INTERPLAY BETWEEN SYMPATHETIC SIGNALING AND INFLAMMATION IN ASEPTIC LOOSENING OF HIP JOINT REPLACEMENT

Article 3

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Interplay between sympathetic signaling and inflammation in aseptic loosening of hip joint replacement

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Abstract

Inflammation is a common symptom in joint disorders such as rheumatoid arthritis, osteoarthritis (OA) and implant aseptic loosening (AL). The sympathetic nervous system is well known to play a critical role in regulating inflammatory conditions, and imbalanced sympathetic signaling has been observed in rheumatoid arthritis. In AL it is not clear whether the sympathetic signaling is impacted. In this study we evaluated the systemic and local profile of neuroimmune molecules involved in the interplay between the sympathetic nervous system and the periprosthetic inflammation in hip AL. Our results showed that periprosthetic inflammation does not trigger a systemic response of the sympathetic nervous system, but is mirrored rather by the impairment of the sympathetic signaling locally in the hip joint. Macrophages were identified as key players in the local regulation of inflammation by the sympathetic nervous system in a process that is implant debris-dependent and entails the reduction of both adrenergic and Neuropetide Y (NPY)-ergic signaling. Additionally, our results showed a downregulation of semaphorin 3A (SEMA3A) that may be part of the mechanism sustaining the periprosthetic inflammation. Overall, the local sympathetic signaling emerges as a putative target to mitigate the inflammatory response to debris release and extending the lifespan of orthopedic implants.

Introduction

Osteoarthritis (OA) is one of the most prevalent chronic joint diseases and a major contributor to functional disability and loss of autonomy in older adults (1). It is associated with a substantial economic and social burden, which will be even higher in the upcoming years, with the expect aging of the population (1). Total joint replacement is considered the actual goal standard for the treatment of patients with severe osteoarthritis, providing pain relieve, improving joint function and enhancing patients´ quality of life (2, 3). Unfortunately, total joint replacements can end up failing, mostly due to periprosthetic inflammation featured by sustained chronic inflammatory response initiated by implant degradation products that shed and accumulate in the neighbor tissue (4). This adverse tissue reaction is orchestrated by the large plethora of immune cells of which the macrophage lineage has been shown to be of major relevance (4, 5). It is well established that macrophages differentiation towards M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotype is of major relevance to the inflammation state and/or resolution. In vitro studies have shown that Polymethyl methacrylate (PMMA) and ultra-high molecular weight polyethylene (UHMWPE) implant particles can polarize macrophages to pro-inflammatory M1 phenotype (6-8). The activation of macrophages and other local cells results in the release of pro-inflammatory factors such as cytokines, chemokines, prostanoids, degradative enzymes and reactive oxygen species (5, 9). These factors underlie the chronic inflammatory scenario that may lead to painful synovitis, pathologic fracture of the surrounding bone and impaired function, instability and loosening of the implant (9).

Over the past decades, accumulated evidence has clearly attributed a pivotal role to the sympathetic nervous system and its neurotransmitters in the regulation of chronic inflammatory conditions (10, 11). It has been demonstrated that the activation of the sympathetic nervous system in the context of inflammation results in the release of high amounts of sympathetic neurotransmitters known to induce an anti-inflammatory effect in a context-dependent manner (11, 12). The immunomodulatory effect of sympathetic nervous system can be achieved directly via adrenergic receptors (ADRs) expressed by the immune cells. Two types of ADRs have been characterized, the alpha (A) and beta (B), which were further divided into nine receptors subtypes (A1A, A1B, A1D, A2A, A2B, A2C, B1, B2 and B3)(13). Stimulation of ADRB2 is reported to activate anti-inflammatory mechanisms on immune cells, while stimuli via ADRA activates pro-inflammatory mechanisms (14). Therefore, the overall result will depend on the ADRs family being activated, which in turn depends on the receptors expression profile, and also on the norepinephrine concentration because norepinephrine has a high affinity to ADRA, only binding to ADRB when at high concentrations (14).

The neuropeptide y (NPY), is a neurotransmitters co-released with norepinephrine by the sympathetic nerve fibers that has also been reported to have modulatory effects on the immune cells activity (15). On the context of the immune response, of the five NPY receptors, the Y1 receptor (Y1R) is the most well studied, and has been shown to have a critical role in immunomodulation, as demonstrated for example by the attenuation of inflammation in Y1R knockout mice (16). In healthy human joints, the synovium is richly innervated with both sympathetic and sensory nerve fibers

(17). In rheumatoid arthritis, data obtained in humans and in animal models revealed a deprivation of neuronal derived neurotransmitters in synovium tissue, due to the loss of sympathetic innervation (14, 18-20). Moreover, the extent of this deprivation is correlated with the severity of the inflammation. In fact, comparative studies reported a reduction of sympathetic innervation in synovial tissue of rheumatoid arthritis patients while in OA patients this does not occur (18). In our previous work, we reported similar absence of sympathetic nerve fibers in periprosthetic tissues from AL patients while, again, in OA patients this does not occur (21). In view of this evidence, the sympathetic activity is affected by the intensity of inflammation taking place in the joint.

However, it is still unknown whether in periprosthetic inflammation associated to the release of debris from orthopedic implants occurs a complete shutdown of the sympathetic activity without any rescue mechanisms, and if the observed alterations are restricted to the joint or are also reflected at systemic level.

In this study we evaluated the systemic and the local profile of neuroimmune molecules involved in the interplay between sympathetic nervous system and the inflammatory response to the debris released by hip implants in AL. A comparison with OA was performed.

Results

Periprosthetic inflammation does not trigger the activation of the systemic neuroimmune regulatory pathway

Several studies have shown the activation of the systemic sympathetic nervous system in response to pro-inflammatory cytokines as a means to mobilize energy-rich molecules and sustain the inflammatory process (14). In order to investigate the impact of periprosthetic inflammation on the systemic sympathetic nervous system activity, the serum levels of norepinephrine, epinephrine and NPY (markers of sympathetic nervous system activity) were measured in AL patients, OA patients and healthy donors. In addition, the serum levels of cortisol were also assessed in the same groups as an indicator of the Hypothalamus-Pituitary-Adrenal (HPA) axis activity, a system that together with sympathetic nervous system compose the hormonal pathway through which the central nervous system exerts a regulatory control over inflammation (14). No differences were found in the norepinephrine and NPY serum levels between the three groups (Figure 1a). Epinephrine levels were below the detection limit of the used commercial kit. A trend to higher cortisol levels was observed in AL patients when compared with healthy donors (p=0.0658), although no statistically significant differences were found (Figure 1a).

The serum levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, key players in the activation of the sympathetic nervous system and HPA axis in response to inflammation (14), were also measured in AL and OA patients and in healthy donors. The blood levels of IL-6 were higher in AL and OA patients as compared to healthy donors (p<0.001 and p<0.01,

respectively) (Figure 1b). The levels of IL-1β and TNF-α were observed to be below the ELISA kits' detection limits in all groups.

Normal cortisol serum levels in the presence of high IL-6 concentrations are indicative of an inadequate cortisol secretion (22), and the ratio serum cortisol/IL-6 was shown to be the more suitable indicator of the HPA axis activity (23). Therefore, to further evaluate the HPA axis activity the ratio of serum cortisol / IL-6 was calculated. Results showed no differences in this ratio between the groups, although a not statistically significant trend to lower values was observed in AO patients when compared with healthy donors (p=0.0517) (Figure 1c).

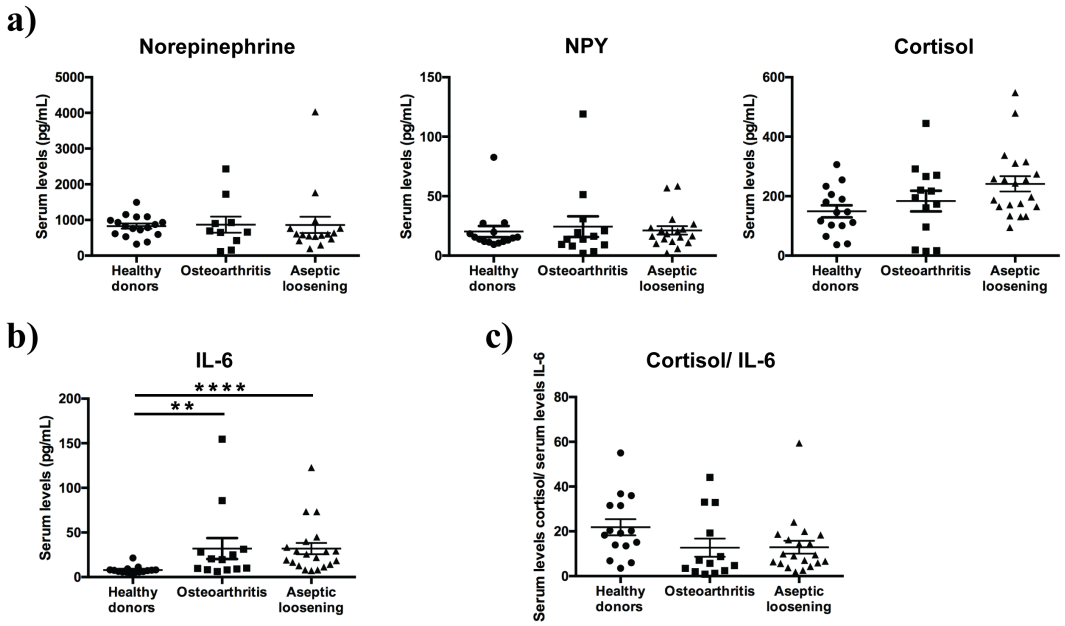

Figure 1. The systemic neuroimmune regulatory pathway was not targeted by periprosthetic inflammation in AL patients

The levels of norepinephrine, NPY and cortisol (a) and the levels of IL-6 (b) were evaluated by ELISA in the serum collected pre-operatively from OA and AL patients, and from healthy donors. The ratio serum cortisol/IL-6 was calculated (c). Results are presented as mean ± SEM, n= 13-15 for healthy donors and AO patients and n=14-20 for AL patients. ** $p< 0.01$; **** $p< 0.001$.

Local sympathetic signaling is impaired in macrophages in periprosthetic tissues from AL patients

Locally, the sympathetic nervous system is known to modulate the inflammatory response throughout adrenergic and NPY-ergic signaling (14). In order to explore the effect of the sustained release of debris from implants on the local sympathetic immune-regulation the expression of tyrosine hydroxylase (TH), ADRA1, ADRA2A and ADRB2, as well as of NPY and Y1R by macrophages, B and T cells (immune cells previously identified, in the same samples used in the present study, as the most prevalent in periprosthetic tissues and in OA synovial membrane (21)) was analyzed in periprosthetic tissues and compared with OA synovial tissues. Macrophages in OA synovial tissues were found to express TH, ADRA1 and ADRB2, but not the macrophages present in periprosthetic tissues (Figure 2a). ADRA2A was expressed by macrophages in both tissues (Figure 2a). Macrophages in OA synovial tissues also stained positively for NPY but not macrophages in AL tissues (Figure 2b).

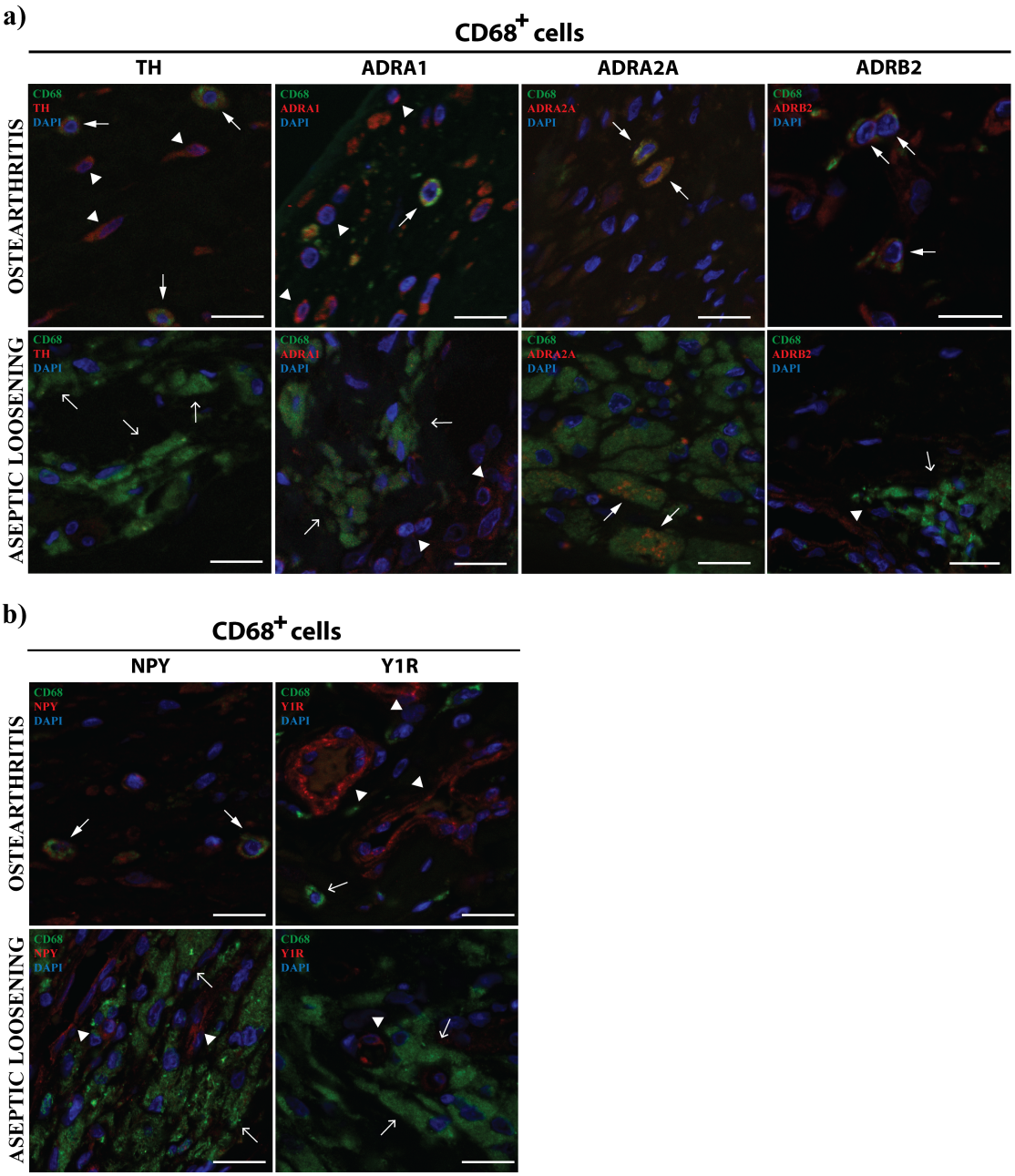

Figure 2. Macrophages in AI periprosthetic tissues do not express TH or ADRB2
The expression of TH, ADRA1, ADRA2A and ADRB2 (a) and the expression of NPY and Y1R (b) by macrophages ($CD68^+$) was evaluated in periprosthetic tissues from AL patients and in synovial tissues from OA patients through double immunohistochemistry staining. Macrophages expressing TH, ADRA1, ADRA2A, ADRB2 or NPY are highlighted with triangle head white arrows. Simple head white arrows indicate macrophages and white arrowheads highlight TH, ADRA1, ADRB2, NPY and Y1R staining in cells other than macrophages (positive control). Scale bar= 20 μm.

Interestingly, the activation of ADRB2 in macrophages has been shown to promote a preferential differentiation of macrophages towards anti-inflammatory M2 phenotype over the pro-inflammatory M1 phenotype (24). However, to our knowledge, the expression profile of ADRB2 in M1 and M2 macrophages was never described. Here we show that in vitro macrophages display a lower ADRB2 mRNA expression levels in M1 as compared to M2 phenotypes ($p<0.05$) (Figure 3). The in vitro analysis of ADRA1 and ADRA2 mRNA expression in M1 and M2 macrophages showed very low expression values, which were in some samples even bellow the detection limit. Still, from the results we were able to achieve, no differences were found in the ADRA1 and ADRA2 mRNA expression between M1 and M2 macrophages (data not shown).

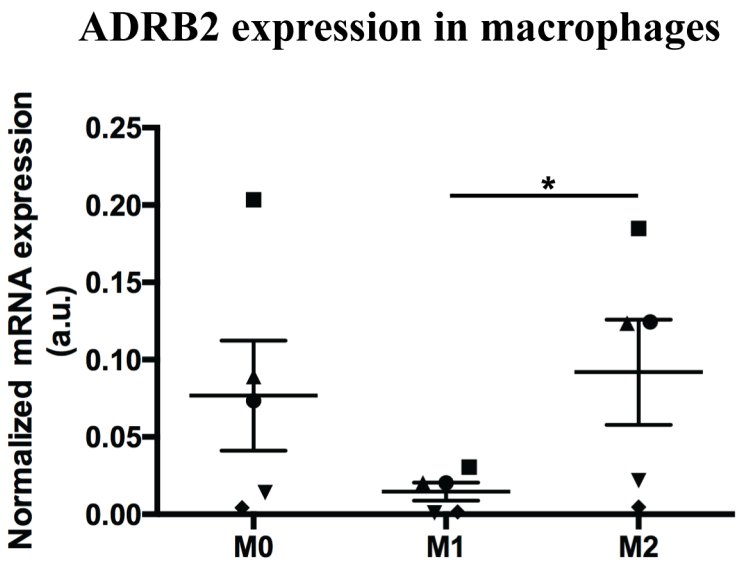

Figure 3. The in vitro expression of ADRB2 is lower in M1 as compared with M2 macrophages
The in vitro mRNA expression of ADRB2 was evaluated in Mo, M1 and M2 macrophages phenotypes. Results are represented as mean ± SEM, for n=5 per group. Each symbol represents macrophages obtained from one specific blood donor. * $p< 0.05$.

T cells were found to express ADRA1 both in periprosthetic tissues and OA synovial membrane, but not ADRA2A and ADRB2 (Figure 4a). T cells were also expressing TH in OA but not in AL tissues. B cells were not expressing the adrenergic markers investigated neither in AL nor in OA (Figure 5a) and the expression of NPY and Y1R was absent from T and B cells in both pathologic conditions (Figure 4b and 5b).

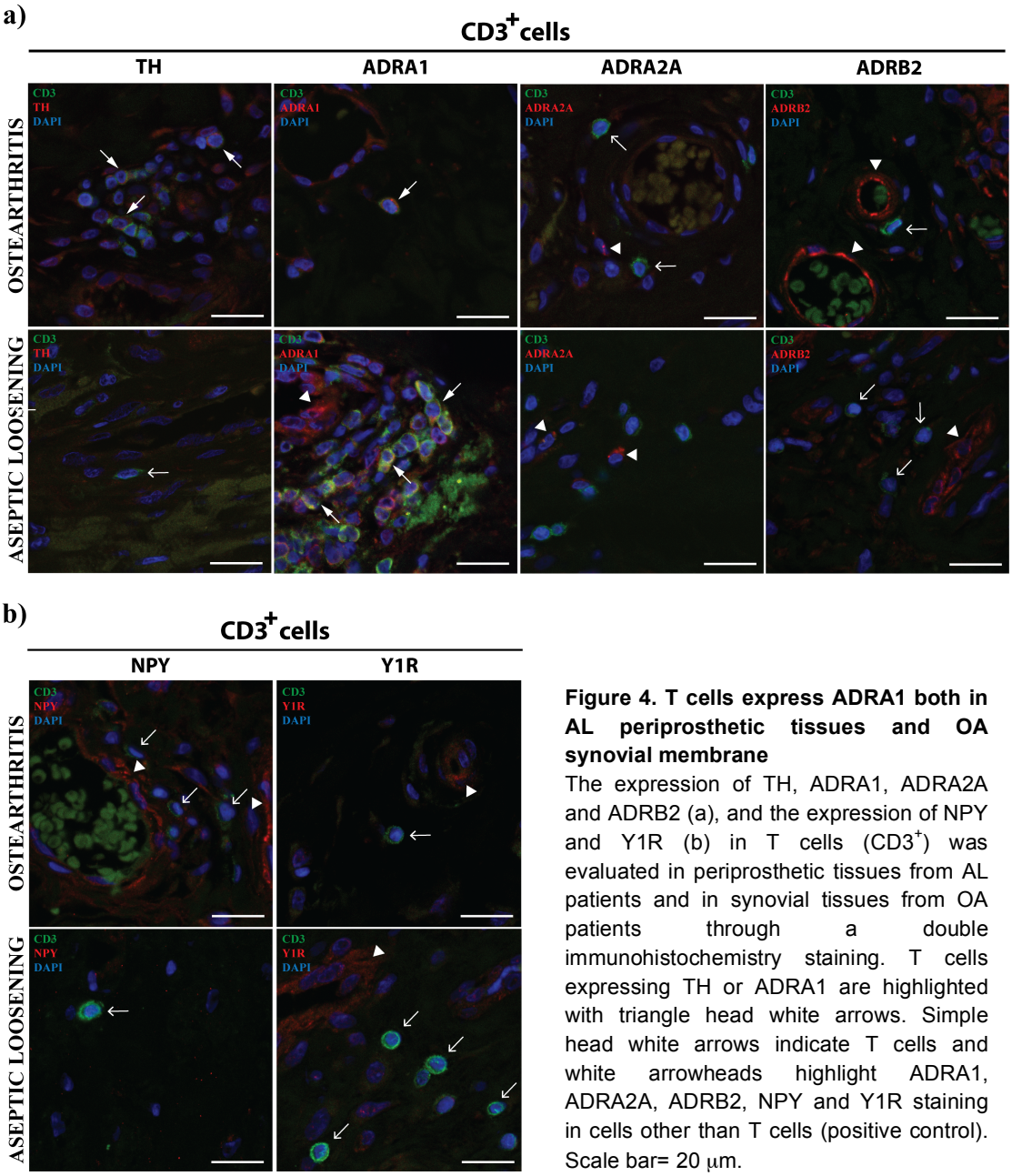

Figure 4. T cells express ADRA1 both in AL periprosthetic tissues and OA synovial membrane
The expression of TH, ADRA1, ADRA2A and ADRB2 (a), and the expression of NPY and Y1R (b) in T cells ($CD3^+$) was evaluated in periprosthetic tissues from AL patients and in synovial tissues from OA patients through a double immunohistochemistry staining. T cells expressing TH or ADRA1 are highlighted with triangle head white arrows. Simple head white arrows indicate T cells and white arrowheads highlight ADRA1, ADRA2A, ADRB2, NPY and Y1R staining in cells other than T cells (positive control). Scale bar= 20 μm.

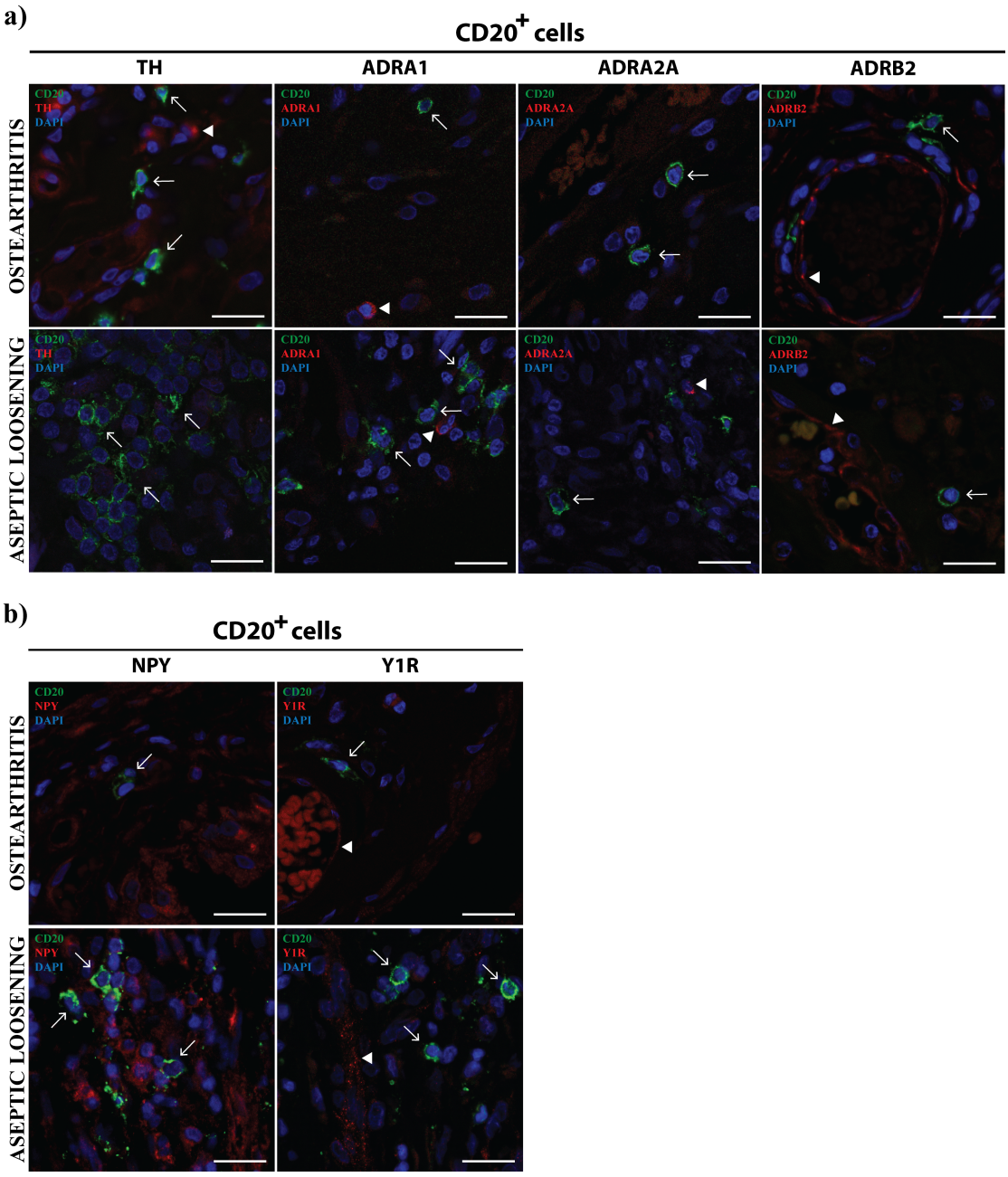

Figure 5. B cells in AL periprosthetic tissues and OA synovial membrane do not express TH, ADRA1, ADRA2A, ADRB2, NPY or Y1R

The expression of TH, ADRB2, ADRA1 and ADRA2A (a), and the expression of NPY and Y1R (b) in B cells (CD20+) was evaluated in periprosthetic tissues from AL patients and in synovial tissues from OA patients through double immunohistochemistry staining. White arrows indicate B cells and white arrowheads highlight positive staining for TH, ADRA1, ADRA2A, ADRB2 and Y1R in cells other than B cells (positive control). Scale bar= 20 µm.

The absence of sympathetic innervation in periprosthetic tissues from AL patients is not mediated by the classic regulators of the innervation pattern.

In a previous work (Vasconcelos et al. 2016, reference 21), using the same samples that were used in this study, we reported an unbalanced innervation pattern in hip periprosthetic tissues, mirrored by the lack of sympathetic nerve fibers. Here, in order to investigate the molecular mechanism underlying this effect, we evaluated the mRNA expression of the classical neurotrophins NGF and BDNF, of the nerve repellent molecule SEMA3A, and of the sympathetic nerve repellent factors SEMA3C and SEMA3F in the AL and OA joint tissues. No differences were found in the mRNA expression levels of NGF and BDNF between periprosthetic tissues and OA synovial membrane (Figure 6a). Interestingly, the mRNA levels of SEMA3A, SEMA3C and SEMA3F were found to be decreased in periprosthetic tissues when compared with OA synovial tissues (SEMA3A: $p< 0.01$; SEMA3C and SEMA3F: $p< 0.05$) (Figure 6b).

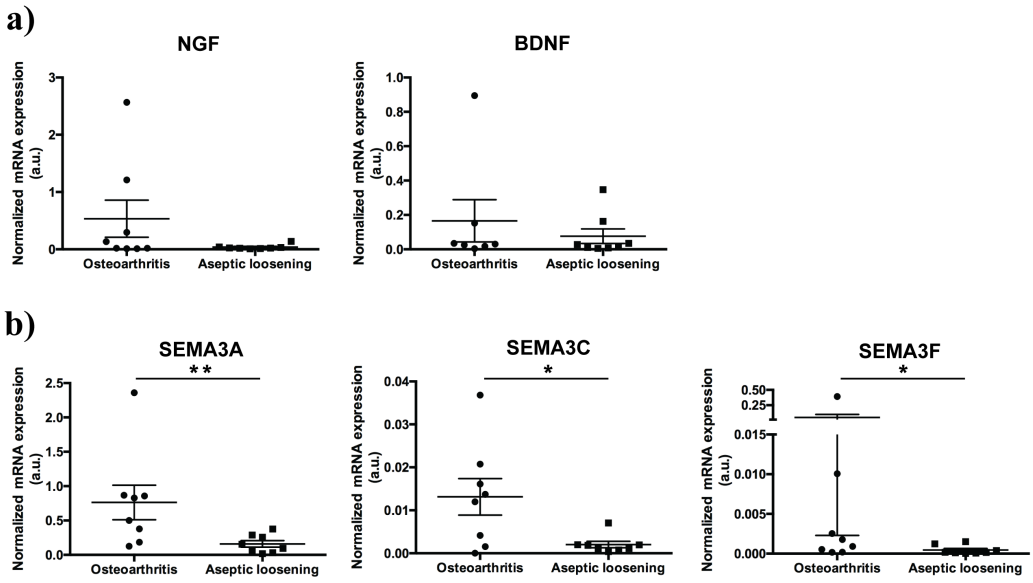

Figure 6. Neurotrophins and semaphorins expression in AL periprosthetic tissues and OA synovial membrane.

The mRNA levels of neurotrophins NGF and BDNF (a), and semaphorins SEMA3A, SEMA3C and SEMA3F (b) were assessed in OA synovial membrane and periprosthetic tissues from AL patients. Results are represented as mean ± SEM, for n=8-9 per group. ** $p< 0.01$, * $p< 0.05$.

Discussion

In this study we show that the periprosthetic inflammatory response is not decoded by the sympathetic nervous system at systemic levels, but is mirrored locally in the hip joint by the impairment of adrenergic and NPY-ergic signaling in macrophages.

Pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 released by local immune cells were identified as key players in the central activation of sympathetic nervous system (14). Moreover, implant debris were shown to promote the in vitro release of pro-inflammatory cytokines by lymphocytes (25) and macrophages (26), and in OA an increase of TNF-α in synovial membrane and blood and of IL-6 in synovial fluid and blood was reported (27). In this study, the comparison of the IL-1β, IL-6 and TNF-α profile showed an increase in the serum levels of IL-6 in AL and OA patients (in comparison with healthy donors) but no differences between AL and OA. Such results highlight IL-6 as a main mediator of the systemic inflammatory response in the studied joint disorders. Still, these increased blood levels of IL-6 were not reflected in an increased systemic sympathetic tone neither in AL nor in OA patients, suggesting the absence of regulation of the inflammatory response by the systemic sympathetic signaling, even in scenarios in which debris are being released from implants. Moreover, the evaluation of the cortisol blood levels and the analysis of the cortisol/ serum IL-6 ratio, strong indicators of HPA axis activity (23), showed no significant differences between the three analyzed groups, suggesting no impact in the HPA axis activity.

The combined lack of activation of systemic sympathetic signaling and HPA axis supports the absence of a systemic control of the inflammatory reaction in both OA and AL conditions.

Within the inflammation site, sympathetic nervous system can directly influence immune cells via the ADRs expressed on their cell surface (28). The sympathetic immunomodulatory effects are known to be dependent on parameters such as the ADRs profile and the catecholamines concentration. The stimulation of ADRA, sensitive to low concentrations of norepinephrine, are described to activate pro-inflammatory mechanisms, while the action on the ADRB2, sensitive to high concentrations of norepinephrine, activates anti-inflammatory mechanisms (14). The sympathetic influence on immune function can also be exerted by other neurotransmitters co-released with norepinephrine, such as NPY (15). In order to understand the impact of debris release in the local sympathetic control of the inflammatory response we investigated both the adrenergic and the NPY-ergic signaling in immune cells. In the follow up of our previous work (21), the expression of TH, ADRA1, ADRA2A and ADRB2 (adrenergic markers) and of NPY and Y1R (NPY-ergic markers) in macrophages, B and T cells, the main immune cell types which we have previously identify in AL periprosthetic tissues and in OA synovial membrane (21) were investigated by immunohistochemistry.

Our immunostaining results showed that TH, ADRA1, ADRB2 and NPY were expressed by macrophages in OA synovial tissues but are absent in macrophages from periprosthetic tissues. These results show that both the adrenergic and the NPY-ergic arms are implicated in the local regulation of joint diseases inflammation by the sympathetic nervous system, indicating

that such local regulation is sensitive to debris released from orthopedic implants and reinforce macrophages as key cellular players in the process. Moreover, the absence of TH and ADRB2 in AL macrophages suggests a lack of sympathetic-mediated anti-inflammatory mechanisms in response to periprostetic inflammation. ADRA were found to be differentially expressed in OA and AL macrophages suggesting a debris-dependent activity of pro-inflammatory mechanisms. In OA the pro-inflammatory activity was found to be mediated by both ADRA1 and ADRA2 while in AL only ADRA2 were found.

We have previously shown that macrophages were more abundant in AL periprosthetic tissues than in OA synovial membrane, and that these cells had a distinct distribution in the two conditions: while macrophages in OA were confined to lining layer, in AL were found infiltrating the periprosthetic tissues (21). Still, the underlying biological meaning of such different macrophage amount and distribution in the local regulation of inflammation is not understood. An increased expression of pro-inflammatory M1 macrophages was found in rheumatoid arthritis patients (29, 30) and in AL periprosthetic tissues when compared with OA synovial membrane (6). In vitro, IL-4 and IL-10 were shown to counteract M1 macrophages polarization induced by PMMA and UHMWPE implant particles, promoting macrophages polarization towards a M2 anti-inflammatory phenotype and inhibiting the inflammatory process (6-8, 31). In orthopedic implants the increased M1/M2 ratio could be responsible for the perpetuation of inflammation, increasing osteolysis and eventually leading to implant failure. Still, very little is known regarding the underlying mechanisms of macrophage differential polarization

in the presence of implant wear debris. Previously, it was demonstrated that the stimulation of ADRB2 in macrophages promotes their differentiation towards an M2 anti-inflammatory profile and serves as a mean to prevent hyper-inflammation (24). In our study we observed that only OA macrophages, and not AL macrophages expressed ADRB2. Following, we decided to assess whether macrophages polarization in M1 or M2 could impact the ADRB2 expression profile. For this aim, an in vitro experiment exploring ADRB2 expression in M1 and M2 macrophages was performed. The results from this experiment showed that the expression of ADRB2 is lower in M1 pro-inflammatory phenotype macrophages than in M2, suggesting that the impairment of ADRB2 signaling observed in macrophages in AL tissues might be involved in the harshness of the periprosthetic inflammation.

In regard to the NPY-ergic regulation, NPY produced by immune cells during inflammation is known to modulate the cellular activity through autocrine or paracrine actions (15, 32), and has been shown to decrease the expression of the pro-inflammatory TNF-α by macrophages after stimulation with LPS (33) and to increase the expression of the anti-inflammatory TGF-β1 (34), contributing to ameliorate the inflammatory response. Moreover, it was recently suggested that NPY promotes the differentiation of macrophages towards a M2 anti-inflammatory phenotype (35).

Altogether, the observed lack of TH, ADRB2 and NPY expression in macrophages of AL periprosthetic tissues may input a preferential differentiation of macrophages towards a pro-inflammatory phenotype, increasing the M1/M2 ratio, which may underlie the perpetuation of

inflammation and an increased osteolysis leading to implant failure. Although several immunomodulatory roles of NPY were suggested to be achieved through Y1R (15), the expression of this receptor was not observed neither in macrophages in periprosthetic tissues nor in OA synovial membrane, suggesting the enrolment of other NPY receptors (namely Y2R and Y5R) in the NPY regulation of macrophages activity (32, 36).

In regard to T cells, ADRA1 was found to be expressed by these cells in both OA and AL. This receptor has been described as an inhibitor of T cells proliferation (37). Considering the fact that our results show the expression of ADRA1 by mature T cells ($CD3^+$) in both AL and OA tissues, a different putative role of ADRA1 in T cells-mediated inflammation, other than the inhibition of proliferation, which is independent of particles released by the implants is suggested. As observed in macrophages, T cells were found to express TH in OA but not in AL tissues, supporting the higher ability of non-neuronal synthesis of catecholamines in OA synovial membrane when compared to AL periprosthetic tissues.

Evidence showed a repulsion of sympathetic nerve fibers from inflamed tissues that cause the loss of anti-inflammatory neurotransmitters and allow the establishment of a privileged pro-inflammatory area (18, 38-40). In a previous work, performed in the same samples used in the present study, we have also reported the repulsion of sympathetic innervation from periprosthetic tissues in AL patient but not from synovial membrane in OA patients (21).

Here, we investigate the putative involvement of well-studied neurotrophic factors, NGF and BDNF, of the nerve repellent molecule SEMA3A, and of

the sympathetic nerve repellent factors SEMA3C and SEMA3F in this specific innervation pattern. No differences were found in the expression levels of NGF and BDNF between AL periprosthetic tissues and OA synovial membrane, and the expression of the chemorepellent molecule SEMA3A, SEMA3C and SEMA3F was in fact lower in periprosthetic tissues as compared with OA synovial membrane. Such results indicate that the most well studied molecules involved in the modulation of the innervation pattern were not responsible for the repulsion of the sympathetic innervation from periprosthetic tissues of AL patients. Considering the fact that SEMA3A has also been described as having an immunomodulatory role (41-43), the observed decrease SEMA3A expression may be part of the mechanism underlying the different inflammatory profile between OA synovial membrane and periprosthetic tissues. In fact, decreased levels of SEMA3A were observed in synovial tissues from rheumatoid arthritis patients compared to synovial tissues in OA patients, and the reduction of SEMA3A expression was correlated with rheumatoid arthritis exacerbation (44). Moreover, overexpression of SEMA3A reduced inflammation in a mouse model of collagen-induced arthritis (45), further supporting SEMA3A as an important immunomodulatory molecule.

Overall, in this work we show that periprosthetic inflammation in AL does not trigger a systemic response of the sympathetic nervous system but enforces the impairment of the local sympathetic signaling as a putative mean to enable the perpetuation of the inflammatory state. Macrophages were highlighted as key cellular players in the local regulation of inflammation by the sympathetic nervous system in a process that is implant wear debris-

dependent and entails the reduction of both the adrenergic and the NPY-ergic signaling. The absence of TH, ADRB2 and NPY expression in macrophages in periprosthetic tissues from AL patients may underlie a preferential differentiation of macrophages towards a M1 pro-inflammatory phenotype, promoting inflammation and increased osteolysis that leads to implant failure. The local sympathetic signaling emerges, therefore, as a putative target to mitigate the inflammatory response to debris release and extending the lifespan of orthopedic implants.

Methods

Samples

Biological samples were collected from three groups of patients. Group 1 was constituted by twenty patients (20 hips) undergoing hip revision surgery due to AL, after exclusion of infection, recurrent dislocation and periprosthetic fractures. All patients had a metal-on-polyethylene coupling and eleven prosthesis were cemented. All acetabular components were revised and in five of those the femoral stem was also exchanged. In four patients metallosis was observed and the mean time to revision was 120.05±65.8 months. Group 2 was constituted by fifteen patients (15 hips), submitted to primary hip replacement surgery for primary OA, after excluding patients with a known history of inflammatory or neoplastic diseases. Group 3 was constituted by 15 healthy volunteers (i.e. without known osteoarticular or systemic diseases). Demographics from Groups 1-3 are presented in table 1.

Serum was obtained from the blood collected, during the morning, previously to surgery in Groups 1 and 2, and in Group 3, and kept at -80ºC until analyses. Synovial tissue was removed during the surgical procedure in Group 2 patients, and periprosthetic tissue was collected in Group 1.

This study was approved by the Ethics Committee of Centro Hospitalar São João and all patients signed an informed consent to the use of their samples for research purposes. All the procedures were in accordance with the Helsinki Declaration of 1975, as revised in 2000.

	N	Age (years) Mean (±SD)	p	Gender N of male/female	p
Group 1 **Revision Surgery due to AL**	20	70.35 ± 11.4	0.119	6/14	0.537
Group 2 **Primary Surgery due to OA**	15	63.33 ± 14.5		6/9	
Group 3 **Healthy Donors**	15	32.87 ± 3.4	<0.001*	9/6	

Table 1. Demographic data on included patients
AL- Aseptic loosening; OA- Osteoarthritis. *Comparing Group 3 with both Group 1 and Group 2.

ELISA

The levels of Epinephrine/Norepinephrine, NPY and Cortisol were measured in the serum obtained from AL patients, OA patients and healthy donors, using ELISA kits from Abcam plc (Cambridge, UK; catalog number: AB108665), Merck KGaA (Darmstadt, Germany; catalog number: EZHNPY-25K) and Abnova (Taoyuan, Taiwan; catalog number: KA1877) respectively, according to the manufacturers' protocols. The serum levels of interleukin (IL)-1β, IL-6 and Tumor necrosis factor alpha (TNF-α) were also measured in the same groups, using ELISA kits from BioLegend (CA, USA; Catalog number: 430507).

Monocyte isolation and macrophage differentiation

Human monocytes were isolated from healthy blood donors and differentiated into macrophages (46). Briefly, 10^6 monocytes/mL/3,8cm^2 were cultured for 10 days in RPMI1640 medium, supplemented with 10% Fetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin, in the absence of Macrophages colony-stimulating factor (M-CSF) or other exogenous factors. 10 ng/mL LPS (Sigma-Aldrich) or IL-10 (ImmunoTools, Friesoythe, Germany) were added, for additional 72h, to polarize macrophages towards M1 or M2

phenotype, respectively. Unstimulated macrophages (M0) were maintained with renewed medium.

Double immunofluorescence staining

The expression of markers of the sympathetic nervous system by immune cells (macrophages, B cells and T cells) was evaluated through a double immunofluorescence staining analysis. The collected tissues were formaldehyde-fixed and processed for paraffin embedding, and cross-sections of 3 μm thickness were cut in the microtome (RM2255, Leica Biosystems). Deparaffinized and dehydrated sections were placed in antigen retrieval for 20 min at 97ºC (10 mM citrate buffer, pH 6.0). After quenching endogenous fluorescence with 0.1% $NaBH_4$ and 100 mM NH_4Cl, sections were incubated with blocking buffer (10% FBS, 1% Bovine serum albumin (BSA), 0.2% Triton X-100).

Simultaneous incubation of each primary antibody against immune cells (antibodies mouse anti-CD68 (clone 514H12, dilution 1:100, Novocastra, UK), anti-CD20 (clone L26, dilution 1:100, Cell Marque, USA), anti-CD3 (clone PS1, dilution 1:100, Biocare Medical, USA)) with each primary antibody against sympathetic markers (antibodies rabbit anti-TH (dilution 1:100, Merck KGaA, Darmstadt, Germany), anti-ADRA1 (dilution 1:100, Abcam, USA), anti-ADRA2A (dilution 1:200, Abcam, USA), anti-ADRB2 (dilution 1:100, Proteintech, USA), anti-NPY (dilution 1:1000, Sigma-Aldrich, USA) or anti-Y1R (dilution 1:500, Immunostar, USA)) were performed overnight at 4ºC.

For signal detection, tissue sections were incubated for 1 hour at room temperature (RT) with a mixture of anti-rabbit Alexa Fluor 568 antibody and

anti-mouse Alexa Fluor 488 antibody (1:1000 dilution, Life Technologies, USA), incubated with DAPI and then mounted with Fluoroshield Mounting Medium (Abcam, USA). Immunostaining images were acquired on the confocal Leica TCS SP2 AOBS (Leica Microsystems, Germany) and Leica TCS SP5 microscope (Leica Microsystems, Germany).

Gene expression analysis

Synovial tissues were homogenized in liquid nitrogen using a mortar and pestle to preserve RNA integrity. RNA from synovial tissues and from macrophages was extracted and purified using TRIzol (Invitrogen, UK) and Direct-zol™ RNA MiniPrep (ZYMO Research, USA), according to the manufacturers' instructions.

RNA purity was estimated from the ratio of absorbance readings at 260 and 280 nm and only ratio between 1.8 and 2 were accepted. RNA quality was verified in agarose gel and RNA concentration was determined in a NanoDrop spectrophotometer (NanoDrop™ 1000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, Delaware, USA NanoDrop). RNA was reverse transcribed using the SuperScript^{TM} First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA, USA).

The transcriptional levels of ADRA1A, ADRA1B, ADRA1D, ADRA2A and ADRB2 in macrophages and neurotrophins (Nerve growth factor (NGF) and Brain-derived neurotrophic factor (BDNF)) and semaphorins (SEMA3A, SEMA3C and SEMA3F) in the AL and OA tissues were evaluated by quantitative real time PCR (qRT-PCR) in the CFX96 Touch Detection System

(Bio-rad, USA). β2 microglobulin (B2M) was used as reference gene for internal normalization. The primers used were as follows: ADRA1A sense primer: 5'-TCAT GTACTGCCGCGTCTAC-3'; ADRA1A antisense primer: 5'-GGGCGTTTTTCCGATGG ATG-3'; ADRA1B sense primer: 5'-CTCTACCGCTTGGCTCCTTGT-3'; ADRA1B antisense primer: 5'-GGAGCATGGGTAGATGATGGG-3'; ADRA1D sense primer: 5'-TCT CCCGTGAGAAGAAAGCG-3'; ADRA1D antisense primer: 5'-CGGGAACAAGGAGCCG AG-3'; ADRA2A sense primer: 5'-ATCCTGGCCTTGGGAGAGAT-3'; ADRA2A antisense primer: 5'-TCTCAAAGCAGGTCCGTGTC-3'; ADRB2 sense primer: 5'GGACTTCCATTG ATGTGCTGT -3'; ADRB2 antisense primer: 5'-GTCAGCAGGCTCTGGTACTTG-3'; NGF sense primer: 5'-AGCGCAGCGAGTTTTGG-3'; NGF antisense primer: 5'-GCTGCTCCCT TGGTAAACTG-3'; BDNF sense primer: 5'- GATGCTCAGTAGTCAAGTGCC-3'; BDNF antisense primer: 5'-GCCGTTACCCACTCACTAATAC-3'; SEMA3A sense primer: 5'CAG CCATGTACAACCCAGTG-3'; SEMA3A antisense primer: 5'-ACGGTTCCAACATCTGT TCC-3'; SEMA3C sense primer: 5'-ATCCGGTCCTGATCTTCATC-3'; SEMA3C antisense primer: 5'-CAGCCCCAAGCAAGAGTTTA-3'; SEMA3F sense primer: 5'-CCAACTACCA GTGGATGCCC-3'; SEMA3F antisense primer: 5'-

GTACACGGCCTGGTACATGA-3'; B2M sense primer: 5'-CCAGCGTACTCCAAAGATTCAG-3'; B2M antisense primer: 5'-AGTCAACTTCAATGTCGGATGG-3'. Relative transcription levels were calculated by comparative threshold cycle quantification (ΔC_t method) using B2M as reference gene.

Statistical analyses

All data were assessed for normal distribution and non-parametric analyses were performed whenever normal distribution was not followed. Hormones and IL-6 levels, and the cortisol/IL-6 ratio were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test. mRNA expression of neutrophins and semaphorins in the synovial tissues was analyzed by Mann-Whitney test and ADRs mRNA expression in macrophages by the Repeated Measures ANOVA followed by Holm-Sidak's multiple comparisons test. Differences were considered at the significant level of $p<0.05$. All data are expressed as mean ± SEM. Statistical analyses were performed using the software Prism 6, GraphPad software, San Diego, CA, USA).

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Authors contributions

MRS, DMV, NN and DL carried out the retrieval of synovial tissues and blood samples. MRS, CJA and DMV performed ELISA quantifications. CJA, MRS and RH performed co-staining analyses. CJA and MRS executed gene expression analysis and all statistical analyses. MJO performed monocytes isolation and differentiation into macrophages. MRS, CJA, ISA, GC and ML conceived of the study, and participated in its design and coordination. CJA and MRS prepared the first manuscript draft. ML and ISA worked in the final manuscript, and all authors read and approved it.

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CHAPTER V
CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Biological events leading to hip osteoarthritis and aseptic loosening are yet to be fully detailed, with our intervention and management of both primary hip osteoarthritis (HOA) and failed total hip arthroplasty (THA) remaining reactionary and palliative. Most of our medical intervention happens when there are established radiographic changes. At this time significant damage has occurred both at joint level in primary cases and at the bone stock in failed THA (1, 2).

Inflammation is common to osteoarthritis (OA) and aseptic loosening (3, 4). HOA is characterized by inflammatory and immune cell infiltration and cytokine secretion (4, 5), that leads to a vicious circle of cartilage degradation and chronic inflammation (6). After THA, inflammation is a response to released wear debris and particles from the implants, which can initiate important immunological reactions, that eventually cause implant failure (7)

Over the past years, accumulated evidence has clearly attributed a pivotal role to the sympathetic nervous system and its neurotransmitters in the regulation of chronic inflammatory conditions (8, 9). Current literature reports on the relationship between innervation and inflammation in osteoarthritic synovial tissues (10), but the influence of the neuroimmune axis in biological events leading to aseptic loosening has been poorly explored (11).

In this context, there is a growing interest in understanding the biological response to both HOA and failure of THA so that new therapeutic strategies can be developed.

In chapter II we performed the first systematic review on neuroimmune expression in HOA patients using data from studies in humans (12). This

study allows an integrated approach of the changes caused by HOA, both at a local and systemic level, contrary to most available studies that focus solely on the actions of a small number of molecules in a specific environment (bone, cartilage, synovial tissue).

A deregulation in the balance between pro-inflammatory and anti-inflammatory cytokines was reported. Interleukin (IL)-6 and tumor necrosis factor (TNF)-α were found to be significantly increased both locally and systemically (12). These increases have been described previously in patients with HOA, as well as their pro-inflammatory and cartilage degradation effects (13, 14). IL-10, a known anti-inflammatory cytokine, is decreased at a systemic level, compatible with a pro-inflammatory state in HOA, but increased locally, which is interpreted as a local attempt to control the inflammatory process (15). Transforming growth factor (TGF)-β was also increased at a local level in HOA patients, but no systemic differences could be found.

In what concerns the influence of the neuroimmune axis in biological events leading to HOA, only two articles were found regarding neuropeptides expression in HOA comparing with healthy subjects (16, 17), which is representative of the lack of available information in this area. Nerve fibers and increased levels of both calcitonin gene-related peptide (CGRP) and substance P (SP) were found in the OA synovial membrane when compared to normal subjects (16, 17), mostly in a perivascular location, a fact also found in papers studying the influence of nerve fibers and neuropetides in bone metabolism (18). Both SP and CGRP are known to play a significant role in pain, as well as contributing to the inflammatory process and cartilage

degradation in both HOA and inflammatory diseases as rheumatoid arthritis (RA) (19, 20). SP was reported to play a pro-inflammatory and catabolic role in the development of OA (21), by increasing the concentrations of IL-1, IL-6 and TNF (22), while CGRP and sympathetic neurotransmitters present an anti inflammatory role by inhibiting these cytokines actions (23, 24). These findings are in accordance with previous works stating the importance of these neuropeptides in the modulation and regulation of the inflammatory process in OA (25, 26). Despite the alterations described above, our review highlights the lack of a clear analytical profile of HOA, both locally and systemically. In addition, our understanding of the influence of the neuroimmune axis in the response to implantable materials, and its influence in the biological processes leading to its failure is still very limited.

In chapter III we addressed the local inflammatory response and neuroimmune profile of aseptic loosening patients and compared it with patients with clinical end stage HOA. This was the first study performing this analysis in the hip of human patients, and brought a new insight into the role of the neuroimmune axis in the hip of HOA and aseptic loosening patients. To our knowledge, only two articles had previously addressed the local innervation profile of patients with aseptic loosening, and presented contradictory results (27, 28). Our results demonstrated different immune responses, with macrophage presence being more intense, and distribution more dispersed in aseptic loosening compared with HOA patients. Macrophages presence is a response to the presence of wear particles (29, 30), and promotes the expression of inflammatory cytokines that can lead to osteolysis (31).

Regarding the inflammatory profile no differences were found in pro-inflammatory cytokines between the two groups. IL-10 was locally increased in both groups, with a tendency to a significant increase in the aseptic loosening group. A similar finding was reported when comparing HOA with healthy patients in our systematic review. The same interpretation, regarding a local attempt to control the inflammation in aseptic loosening patients can be made, a feature also described by other authors (32).

TGF-β was significantly increased in synovial messenger ribonucleic acid (mRNA) in OA patients when compared to aseptic loosening, but with increased immunostaining in aseptic loosening when compared to OA. The effects of TGF-β in inflammatory processes are not clear. It can present a dual effect on arthritic diseases and an action dependent on the local existing context (33). TGF-β can contribute to matrix production and chondrocyte proliferation at a local level (34), but also induce pro-inflammatory cytokine expression (35). There is an increased interest regarding its potential therapeutic effects as an immunomodulator (33). Locally this was the main difference found, that led us to investigate if there was any systemic alteration in TGF expression, which was not confirmed. These findings are in accordance with what was reported in our systematic review, where a local increase of TGF, without systematic translation, was also described when comparing HOA with healthy patients (12).

Interestingly, our finding results revealed that HOA patients have distinct local innervation when compared to aseptic loosening patients. Both HOA and aseptic loosening patients present sensory innervation with positive SP and CGRP fibers, but with a different distribution. While in OA patients their

presence is localized and close to blood vessels, in aseptic loosening its distribution is dispersed. We also reported an absence of sympathetic nerve fibers in aseptic loosening tissues when compared to OA, which is a distinct feature in the innervation profile of this group, and the first time this was described. This finding is similar to what is found in RA patients (36), and could, simultaneously, be related to the presence of a persistent inflammatory environment, and a cause associated with its maintenance. Current evidence gives the sympathetic nervous system and its neurotransmitters a pivotal role in the regulation of chronic inflammatory conditions (8, 9), with activation of the sympathetic nervous system in the context of inflammation resulting in the release of high amounts of sympathetic neurotransmitters, like norepinephrine (NE) and neuropeptide Y (NPY), known to induce anti-inflammatory effects (24).

These insights shed light on neuro-immune interplay in both aseptic loosening and HOA and underline the need to better understand this crosstalk to unravel potential mechanisms for targeted-therapies to improve hip joint lifetime and treatment.

In chapter IV we investigated further whether in aseptic loosening joint occurs a complete shutdown of the sympathetic activity without rescue mechanisms, and if the observed alterations are also reflected at systemic level. Additionally, putative local mechanisms underlying the lack of sympathetic innervation were also explored.

Locally we confirmed that the usual effectors for the sympathetic anti-inflammatory action (37, 38), β2-adrenergic receptors (β2-ARs) and NPY, were absent in macrophages in aseptic loosening patients but present in the

OA group. Macrophages were described in chapter III as being the most prevalent immune cell found in local tissues of both THA and AL patients. β2-ARs and NPY-Y1 receptors are known to stimulate M2 macrophages that have a pro-regenerative and anti-inflammatory action when compared to M1 (39, 40). Y1 when activated by NPY also reduces the expression of pro-inflammatory cytokine TNF-α by macrophages (41) and increases the expression of TGF-β (42). The observed lack of β2-ARs and NPY expression in macrophages of aseptic loosening periprosthetic tissues may input a preferential differentiation of macrophages towards a pro-inflammatory phenotype, increasing the M1/M2 ratio, which may underlie the perpetuation of inflammation and increased osteolysis leading to implant failure. Together all these findings are in favor to local reduction of the anti-inflammatory effects performed by the sympathetic system, and the perpetuation of a pro-inflammatory environment in aseptic loosening patients.

Concerning the impact on the systemic sympathetic nervous system activity, there was no difference in the expression of epinephrine, NE and NPY between OA, aseptic loosening and healthy groups. As expected, when comparing the systemic expression of pro-inflammatory cytokines, there was an increase in the OA and aseptic loosening groups versus healthy patients, but no difference between the OA and aseptic loosening groups. These results confirm the local findings in chapter III regarding no differences between groups in local cytokine expression, and fail to demonstrate any systemic consequence of the sympathetic differences seen between the OA and aseptic loosening groups. Also we found no systemic differences in cortisol expression or cortisol/IL6 ratio between groups, which means no

impact in the Hypothalamus-Pituitary-Adrenal (HPA) axis activity. As pointed out in chapter IV, the HPA axis is a system which together with sympathetic nervous system, compose the hormonal pathway through which the central nervous system exerts a regulatory control over inflammation (43).

This lack of systemic impact in the regulation of the sympathetic nervous system, leads us to agree with authors who believe that in humans the pathological responses occurring at the hip seem to be mostly confined to the joint (44).

The study of these processes in humans is important to overcome the fact that most available studies translate data from in vitro or in vivo animal models and compare them to the human situation (45).

In chapter III and IV we used data from analyses of tissues from late HOA or failed THA. Tissues retrieved during surgery represent late stages of a process that, in the majority of cases, reflects a long lag period from the time at which the local homeostasis was first disrupted (3). This contributed to enlighten the changes of end stage HOA and aseptic loosening, describing features and differences that were previously unknown, but did not allow the evaluation of local findings during the development or earlier stages of these diseases.

Another important reflection regards the individual methods found in available works, and their implication in our current knowledge and ability to make assumptions.

In our systematic review there are limitations in sample sizes, with most articles using very few patients. The population used as controls is poorly defined and cannot assure that the comparisons made are not impacted by

these facts (46, 47). The methods for sample collection, processing and analysis are also heterogeneous. For the same reasons a quantitative analysis was not possible. Future studies should focus in better-defined criteria for patient and control selection, sample collection, processing and analysis.

In our experimental works, the sample numbers are similar to the average numbers published in this field (32), and were collected by the same team to minimize selection bias. We recognize that the interpretation of the variability described between groups can be influenced by the biological response to other systemic conditions, which we were not able to eliminate in patients selection. The use of samples from OA patients allows to overcome the lack of access to healthy synovial tissue or well-integrated implants. However, the use of OA tissues as control is a limitation of the majority of studies because two different immune responses are being compared in a pathological context. Future studies must include more defined populations and apply more strict inclusion criteria, regarding the presence of relevant comorbidities with impact on the immune system. An increase in the number of patients treated arthroscopically for non-degenerative conditions of the hip may also allow the collection of healthy samples from synovial membrane, cartilage and bone, and overcome some of the limitations described.

Interventions that would be disease modifying in HOA are elusive for the time being, and joint arthroplasty despite highly successful in relieving symptoms, is not a procedure without significant risks and cost. Future studies should continue to enlighten HOA and aseptic loosening pathophysiology, as the

potential for improved management of HOA and THA failure relies in shifting the treatment paradigm to the earliest stages of the condition's pathogenesis.

Overall, under the scope of this thesis, we described a new pattern of innervation in AL, characterized by the absence of sympathetic activity. This impairment occurs locally at the hip, affecting both adrenergic and NPYergic signaling, without systemic translation. This finding highlights the local sympathetic signaling as a putative target to mitigate the inflammatory response to debris release and consequently extending the lifespan of orthopedic implants.

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